PROTEIN MICROSPHERES FOR CONTROLLED DRUG DELIVERY AND RELATED ANALYSIS OF BIOPOLYMERS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1997

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To my parents, Donald and Alice Kirk.

ACKNOWLEDGMENTS

The unsung heroes of any dissertation are the departmental staff that make graduate education possible. Of the many people who extended themselves to make my time at the University of Florida easier, I especially would like to thank Jackie Hulsey and Joe Rojo on the administrative side as well as Paul Martin and Dr. Lynn Peck on the technical side, for the myriad things they did both large and small.

I am grateful to my committee: Dr. Christopher Batich, Dr. Anthony Brennan, Dr. Edward Hoffmann, Dr. James Seeger and my committee chair, Dr. Eugene Goldberg. The knowledge and guidance they have given me is a debt that I can never repay. Thus, I vow to honor their efforts as educators by passing that knowledge on to those who follow me.

Then there are my fellow students. A friend in law school once remarked to me how peculiar it was that the most incisive journals on the law were written largely by people who are not yet lawyers. Yet, to me, this is the essence of the graduate experience in science and engineering. We, the apprentice masons in the halls of knowledge, study together the foundations and structures already laid down and then

strive to lay on new courses. The list of fellow laborers is too long to recount in full, so I will give mention to a special few and hope that the rest will be understanding. Dr. Khalid Mentak and Dr. Jeanne Quigg have already gone but were both kind to and mindful of the "new kid," while Drew Amery, James "Bubba" Marotta, Dr. Chris Widenhouse, and John Wironen were contemporaries that helped to keep me on track.

Finally, I thank my family and friends for all of their support over the years. You know who you are, I only can hope that you know just how grateful I am.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iv

LIST OF TABLES ix

LIST OF FIGURES xiv

KEY TO SYMBOLS, ABBREVIATIONS, AND ACRONYMS xvii

ABSTRACT xix

1 INTRODUCTION 1

1.1 Clinical Motivation 1
1.1.1 Rheumatoid Arthritis 1
1.1.2 Neoplastic Diseases 5
1.2 Current Treatments 6
1.2.1 Rheumatoid Arthritis Treatments 6
1.2.2 Chemotherapy 8

2 BACKGROUND 10

2.1 Microspheres 10
2.1.1 Applications 10
2.1.2 Design Considerations 13
2.2 Choice of Drug 14
2.2.1 Methotrexate 15
2.2.2 Mitoxantrone 19
2.2.3 DMPL 20
2.2.4 Acid Phosphatase 22
2.3 Choice of Carrier Materials 23
2.3.1 Serum Albumin 25
2.3.2 Hyaluronic Acid 27
2.3.3 Carboxymethylcellulose 29
2.4 Cross Linking 31
2.4.1 Constraints and Options 31
2.4.2 Glutaraldehyde 32
2.4.3 Multivalent Metal Ions 36
2.4.4 Microwave 39

2.5 Processing Considerations 39
2.5.1 Surfactant Vs Steric Stabilization 40
2.5.2 Cross Linking Step 41
2.5.3 Pre Vs Post Synthesis Drug Loading 42
2.6 Summary of Proposed Research 43

3 MATERIALS AND METHODS 45

3.1 Materials 45
3.1.1 Carrier Matrix Materials 45
3.1.2 Cross Linking Agents 46
3.1.3 Drugs 48
3.1.4 Equipment, Materials and Supplies 48
3.2 Methods: Microsphere Synthesis 49
3.2.1 Methotrexate Studies 50
3.2.2 Novantrone[tm] Studies 55
3.2.3 Acid Phosphatase Studies 59
3.2.4 DMPL Studies 62
3.2.5 Miscellaneous MS Synthesis Studies 66
3.3 Methods: Drug Content and Elution 73
3.3.1 Methotrexate Studies 73
3.3.2 Protein Elution 75
3.3.3 Novantrone[tm] Studies 77
3.3.4 Acid Phosphatase Elution Studies 78
3.3.5 DMPL Studies 82
3.4 Methods: *In Vivo* Studies 82
3.4.1 Lewis Lung Carcinoma 82
3.4.2 Murine Ovarian Teratocarcinoma 85

4 RESULTS AND DISCUSSION 87

4.1 Methotrexate Studies 87
4.1.1 MS Synthesis 87
4.1.2 MTX Elution 90
4.1.3 Protein Elution 99
4.2 Novantrone[tm] Studies 106
4.2.1 MS Synthesis 106
4.2.2 Lewis Lung Carcinoma Study 107
4.2.3 Murine Ovarian Teratocarcinoma Study 110
4.3 Acid Phosphatase Studies 114
4.3.1 MS Synthesis 115
4.3.2 Acid Phosphatase Elution 115
4.4 DMPL Studies 123
4.4.1 MS Synthesis 123
4.4.2 DMPL Loading 123
4.5 Miscellaneous MS Synthesis Studies 125
4.5.1 MS Synthesis 125
4.5.2 Protein Elution 127

5 CONCLUSIONS AND RECOMMENDATIONS 131

5.1 Methotrexate Studies 131
5.1.1 Conclusions 131
5.1.2 Recommendations 132
5.2 Novantrone[tm] Studies 134
5.2.1 Conclusions 134
5.2.2 Recommendations 135
5.3 Acid Phosphatase Studies 136
5.3.1 Conclusions 136
5.3.2 Recommendations 136
5.4 DMPL Studies 137
5.4.1 Conclusions 137
5.4.2 Recommendations 137
5.5 Miscellaneous MS Synthesis Studies 138
5.5.1 Hydrophobicity 138
5.5.2 HA and CMC as Carrier Materials 140
5.5.3 a-PEG Cross Linking 141
5.5.4 Microwave Cross Linking 142

APPENDIX 144

A.1 Fluorinated Polyethyleneglycol 144
A.1.1 Introduction 144
A.1.2 Materials and Methods 147
A.1.3 Results and Discussion 150
A.1.4 Conclusions 156
A.2 Silicone Breast Implant Gels 157
A.2.1 Introduction 157
A.2.2 Materials and Methods 159
A.2.3 Results and Discussion 160
A.2.4 Conclusions 163

LIST OF REFERENCES 165

BIOGRAPHICAL SKETCH 173

LIST OF TABLES

Table page

1.1 Numbers of patients per year by gender and by age group diagnosed with rheumatoid arthritis (from all listed diagnoses for those patients) discharged from short-stay hospitals 2

1.2 Criteria and exclusions for diagnosis of rheumatoid arthritis adapted from criteria promulgated by the American Rheumatism Association 4

2.1 Applications of microcarrier technology 11

2.2 Several common components of joint fluid with typical concentrations for normal and rheumatoid joints, adapted from Gatter 25

2.3 Comparison of amino acid residues in human serum albumin to residues in bovine serum albumin, adapted from Haurowitz, Table VIII-2 26

3.1 Compositions for the MS lots produced for the MTX studies using glutaraldehyde to cross link the BSA matrix. All masses in grams 52

3.2 Compositions for the MS lots produced for the MTX studies using ferric nitrate nonahydrate to cross link the BSA matrix. All masses in grams 54

3.3 Compositions for the MS lots produced for the Novantrone[tm] studies. All masses in grams 56

3.4 Compositional variations in microsphere lots produced for the acid phosphatase studies. All masses in grams 62

3.5 Compositions and amounts for the lots produced for use by Ribi ImmunoChem in the DMPL studies. All masses in grams 64

3.6 Lot designations and compositions for studies on HA and CMC as matrix materials and on a-PEG and microwave exposure as cross linking agents for stabilizing BSA MS. All masses in grams 66

3.7 Sample and enzyme concentrations for the first enzyme elution study performed at low temperature 79

3.8 Sample and enzyme concentrations for the second enzyme elution study performed at room temperature 81

3.9 Six treatment groups for the LLC study using both free (dissolved) and bound (lot 9201) Novantrone[tm] 84

3.10 Treatment groups for the MOT study using both free (dissolved) or bound Novantrone[tm] in PBS 86

4.1 Percent yield, mean diameter, drug content and protein content for glutaraldehyde cross linked, methotrexate and sodium methotrexate loaded MS. Mean diameter in microns $\pm$ sample standard deviation (n = 100) 88

4.2 Percent yield, drug content and protein content for ferric nitrate nonahydrate cross linked, methotrexate and sodium methotrexate loaded MS 89

4.3 P values for comparisons of percent elution of initial loading of MTX from glutaraldehyde cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 93

4.4 P values for comparisons of percent elution of initial loading of MTX from ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 94

4.5 P values for comparisons of percent elution of initial loading of Na-MTX from glutaraldehyde cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 96

4.6 P values for comparisons of percent elution of initial loading of Na-MTX from ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 98

4.7 P values for comparisons of percent elution of initial loading of protein from lots with low cross link level using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 100

4.8 P values for comparisons of percent elution of initial loading of protein from lots with high cross link level using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 102

4.9 P values for comparisons of percent elution of initial loading of protein from low ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 104

4.10 P values for comparisons of percent elution of initial loading of protein from high ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference 106

4.11 Percent yield, mean diameter, and drug content results for the MS lots produced for the Novantrone[tm] studies. Mean diameter in microns $\pm$ sample standard deviation, n = 100. Drug content in percent w/w, see text 107

4.12 Percent survival at day 35 by treatment group. 108

4.13 Results of the MOT study by treatment group including total survivors and percent survival at day 90; mean survival time (MST) in days ± standard deviation; and P value by Student's t test for treatment group MST compared to control group MST 111

4.14 Lot descriptions and yield results for lots 9613, 9614, 9616, and 9617. All masses in grams 115

4.15 Acid phosphatase elution study protocol. Lots 9614 and 9616 were post-loaded and lot 9617 was bulk loaded. 116

4.16 Enzyme activity after heat treatment. Enzyme activity in units/mL (± sample standard deviation, n = 3) after 0 and 24 hours rehydration at room temperature for control solutions and rinsed suspension as well as after 23 hours at 5°C and 1 hour at 60°C to 65°C and after 23 hours at room temperature and 1 hour at 95°C to 100°C 121

4.17 Possible causes for loss of enzymatic activity for enzyme loaded onto or into BSA microspheres 122

4.18 Lot composition, yield and mean diameters (μm ± sample standard deviation, n = 100) for the lots prepared for DMPL loading studies at Ribi ImmunoChem, Inc 124

4.19 Uptake of DMPL from ethanol and/or triethylamine (TEA) solution by MS as a percent of initial MS mass 124

4.20 Percent yield results for the miscellaneous MS synthesis studies by lot designation and composition .. 126

4.21 P values for comparisons of percent elution of initial loading of protein from lots cross linked with glutaraldehyde, 9620 and 9641; ferric nitrate, 9637; and microwave, 9709 using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with P ≤ 0.05 indicating a significant difference. 129

A.1 Results of solution analyses done on the solutions used in the animal studies. "Low shear" and "high shear" viscosity were performed with a #40 cone at 3.75/sec. and a #52 cone at 100.0/sec. shear rate, respectively 150

A.2 Results of the cecal abrasion studies comparing 1.1% f-PEG solutions to PBS (negative control), 0.4% HA and 0.7% CMC (positive controls) for various measures of adhesion incidence as defined in the text 155

A.3 Results for the gel flow analysis of silicone gel filled breast implants including two series of both unused and explanted implants. The gel flow index is given as the mean of ten measurements +/- the sample standard deviation 161

LIST OF FIGURES

Figure page

2.1 Chemical structures of methotrexate and of tetrahydrofolate. The high degree of structural similarity is responsible for the antifolate behavior of methotrexate 16

2.2 Schematic of pathways affected by methotrexate (MTX) and polyglutamated methotrexate 17

2.3 Chemical structure of mitoxantrone 20

2.4 Chemical structure of the hyaluronic acid repeat unit 28

2.5 Chemical structures of a) cellulose and of b) carboxymethylcellulose with a degree of substitution of 1. Note the different locations of substitution 30

2.6 Chemical structure of a) glutaraldehyde and b) schematic of the Schiff base reaction of aldehyde with a primary amine 33

2.7 Chemical structure of aldehyde terminate polyethyleneglycol 35

2.8 Schematic representation of the formation of ionic cross links by the chelation of ferric nitrate by pendent carboxyl groups 37

4.1 Elution of MTX from glutaraldehyde cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9621 and 9622 versus 9623 and 9624) for low and high cross link level (odd versus even) 92

4.2 Elution of MTX from ferric nitrate cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9701 and 9702 versus 9703 and 9704) for low and high cross link level (odd versus even) 94

4.3 Elution of Na-MTX from glutaraldehyde cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9628 and 9629 versus 9630 and 9631) for low and high cross link level (even versus odd) 96

4.4 Elution of Na-MTX from ferric nitrate cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9705 and 9706 versus 9707 and 9708) for low and high cross link level (odd versus even) 98

4.5 Elution of protein from BSA MS of low glutaraldehyde cross linked level versus time in hours of exposure to PBS at 37°C for unloaded (9619), MTX loaded (low 9621, high 9623), and Na-MTX loaded (low 9628, high 9630) lots 99

4.6 Elution of protein from BSA MS of high glutaraldehyde cross linked level versus time in hours of exposure to PBS at 37°C for unloaded (9620), MTX loaded (low 9622, high 9624), and Na-MTX loaded (low 9629, high 9631) lots 101

4.7 Elution of protein from BSA MS of low level ferric nitrate cross linking versus time in hours of exposure to PBS at 37°C for unloaded (9638), MTX loaded (low 9701, high 9703), and Na-MTX loaded (low 9705, high 9707) lots 103

4.8 Elution of protein from BSA MS of high level ferric nitrate cross linking versus time in hours of exposure to PBS at 37°C for unloaded (9637), MTX loaded (low 9702, high 9704), and Na-MTX loaded (low 9706, high 9708) lots 105

4.9 Percent survival versus time for the LLC study by treatment group 109

4.10 Percent survival versus time (days) post injection for untreated controls and groups 2, 3, and 4 treated with free drug (NOV) at dosages 250, 125, and 63 μg respectively 112

4.11 Percent survival versus time (days) post injection for untreated control, group 1; pre-loaded MS (lot 9308) groups 5, 6, and 7; and unloaded MS (lot 9306), group 11 113

4.12 Percent survival versus time (days) post injection for untreated control, group 1; post-loaded MS (lot 9307), groups 8,9 and 10; and unloaded MS (lot 9306), group 11 114

4.13 Assayed enzyme activity versus time for control solutions (50 µg/mL of acid phos./PBS) and supernatants from suspensions of lots 9616 and 9617 (approximate suspension concentrations of 150 µg/mL of acid phos./PBS) for elution studies done at 5°C and 24°C 117

4.14 Assayed enzyme activity versus time for control solutions (50 µg/mL acid phos./PBS) and supernatants from suspensions of lots 9614, 9616, and 9617 (approximate suspension concentrations of 150 µg/mL acid phos./PBS) for elution studies done at 24°C 118

4.15 Assayed enzyme activity versus time for control solutions (50 µg/mL acid phos./PBS) and rinsed suspensions of lots 9614, 9616, and 9617 (approximate suspension concentrations of 150 µg/mL acid phos./PBS) for elution studies done at 24°C 119

4.16 Protein elution from microsphere suspension into PBS at 37°C versus time in hours. Lots 9620 and 9641 cross linked with glutaraldehyde; lot 9637 with ferric nitrate; and lot 9709 by microwave irradiation. Normalized to a percentage of initial protein loading in suspension 129

A.1 Structure of perfluorooctane terminated polyethylene-glycol 146

A.2 Viscosity versus shear rate for C8F17 terminated PEG at 0.75% and 0.9% w/v in PBS (#40 cone, 25°C) 151

A.3 Viscosity versus concentration for both autoclaved and non-autoclaved PEG (5 MDa) and C8F17 terminated PEG (35 kDa); nominal shear rate 38/sec. at 25°C 152

A.4 Autoclaving induced degradation in viscous behavior of C8F17 terminated PEG (35 kDa) for 0.8%, 0.9% and 1.0% w/v in PBS (#40 cone, 25°C) 153

A.5 Gel flow index versus implant duration (to the nearest whole year) for Dow Corning implants, series I 162

A.6 Gel flow index versus implant duration (to the nearest whole year) for McGhan implants, series II 163

KEY TO SYMBOLS, ABBREVIATIONS, AND ACRONYMS

BSA	Bovine serum albumin
a-BSA	Lipid free bovine serum albumin
°C.	Degrees Celsius
ca.	Circa
CAB	Celluloseacetatebutyrate
CAB/DCE	A mixture, usually 2.0% weight/volume, of CAB and DCE
$CELD_{50}$	Lethal dose to yield 50% deaths in chick embryos
CMC	Carboxymethyl cellulose
cps	Centipoise
Da	Daltons, unit of molecular weight in gram moles
dL	Deciliter
DCE	1,2 Dichloroethane
DMPL	Detoxified monophosphoryl lipid A
g	Gram
g's	Acceleration of gravity relative to Earth
GFI	Gel flow index, reported in grams/10 minutes
GSDCE	Glutaraldehyde saturated DCE
GST	Glutaraldehyde saturated toluene
HA	Hyaluronic acid
HSA	Human serum albumin
hr	Hours
i.p.	Intra peritoneal
i.t.	Intra turmoral
i.v.	Intra venous
kDa	Kilodaltons
kg	Kilogram
L	Liter
LD_{50}	Lethal dose to yield 50% deaths by specified route of administration
LLC	Lewis lung carcinoma, a mouse cancer model
µg	Microgram
µL	Microliter
µm	Micron
mg	Milligram
mL	Milliliter
min.	Minutes
mOsm	Milliosmoles
MDa	Megadaltons

MOT	Murine ovarian teratocarcinoma, a mouse cancer model
MTX	Methotrexate
Na-MTX	Sodium salt of methotrexate
NOV	Novantrone®, mitoxantrone
NSAID	Nonsteroidal anti-inflammatory drugs
PDMS	Polydimethylsiloxane, silicone polymer
PEG	Polyethylene glycol
a-PEG	Propionaldehyde terminated Polyethylene glycol
f-PEG	Perfluoroalkane terminated Polyethylene glycol
PGA	Poly-L-glutamic acid
PVP	Polyvinylpyrrolidone
RA	Rheumatoid arthritis
sec.	Seconds
S.D.	Standard deviation
S.S.D.	Sample standard deviation
w/v	Weight (mass) of solute used by volume of solvent used
w/w	Weight (mass) of solute used by sum of weight (mass) of solute and solvent used

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

PROTEIN MICROSPHERES FOR CONTROLLED DRUG DELIVERY AND RELATED ANALYSIS OF BIOPOLYMERS

By

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August, 1997

Chairperson: Dr. Eugene P. Goldberg
Major Department: Materials Science and Engineering

Rheumatoid arthritis (RA) is a systemic disorder which manifests itself most notably in the synovial joints. In recent years, methotrexate (MTX), a folate antagonist, has been used with some success for treatment of RA. MTX has a maximum cumulative dose beyond which it becomes dangerous to administer due primarily to liver toxicity. This unfortunate side effect has prompted research into means of delivering MTX to the synovial joint in hopes of achieving greater efficacy with reduced toxicity.

Both MTX and its sodium salt (Na-MTX) were loaded into microspheres (MS) composed of bovine serum albumin (BSA) stabilized by cross linking with dialdehydes or ferric ion. MS were prepared with two levels of drug loading at two different levels of cross linking. MTX loading as high as

46.8% w/w was achieved in the aldehyde cross linking system and as high as 46.3% w/w with ferric ion cross linking. Using Na-MTX, the values were 37.2% w/w and 31.7% w/w respectively.

Both MTX and Na-MTX were elutable from the MS into phosphate buffered saline at 37°C. MTX elution from aldehyde cross linked microspheres was load dependent with ca. 60% eluted by 9 hours at low loading and ca. 60% eluted by 24 hours at high loading. In the ferric ion cross link system, the elution was independent of loading with 50% elution occurring between 20 and 48 hours. Na-MTX elution was independent of drug loading or cross link system with 50% elution occurring in less than two hours in all cases.

Other investigations included mitoxantrone (NOV) and enzyme loaded BSA compositions. NOV-BSA microspheres were prepared at a concentration of 19.3% w/w and were effective in the treatment of murine ovarian tumors. A model enzyme, acid phosphatase, was also successfully incorporated into BSA microspheres and retained initial activity for up to four months on post-loaded spheres. This enzyme also remained active inside the microsphere as demonstrated by a substrate cleavage assay.

CHAPTER 1
INTRODUCTION

1.1 Clinical Motivation

Many diseases require treatment with drugs that have adverse side effects. One means of minimizing those side effects is to deliver these drugs directly to the affected organ and release them in therapeutic doses to the affected tissues. This research was directed primarily at the targeted or localized delivery of methotrexate for use in the treatment of rheumatoid arthritis (RA). Related studies were also devoted to a) the localized delivery of mitoxantrone for chemotherapy in treatment of cancer and b) a preliminary investigation into the delivery of enzymes via protein microspheres.

1.1.1 Rheumatoid Arthritis

Rheumatoid arthritis is usually perceived as a chronic malady of the joints in the elderly, most often women. While it is true that RA afflicts women at approximately three times the rate it afflicts men, Table 1.1 shows that over 9% are under the age of 45 and over 44% are under the age of 65. These data are taken from the patient population

who required hospitalization in short-stay hospitals for the years listed and thus understate the incidence in the population at large. The increases seen between 1979 and 1985 are most likely due to the increasing average age of the American population.

Table 1.1 Numbers of patients per year by gender and by age group diagnosed with rheumatoid arthritis (from all listed diagnoses for those patients) discharged from short-stay hospitals.[28, 42, 58]

Year	Male	Female	Less than 45 years	45 to 64 years old	65 years and older
1985	51,000	172,000	22,000	77,000	123,000
1983	59,000	178,000	22,000	82,000	130,000
1979	56,000	153,000	22,000	87,000	99,000

It is difficult to give an elementary description of rheumatoid arthritis without plagiarizing one of the many authors who have written on this subject, hence this quote from the introductory passage of an excellent review by Krane:[43]

> Rheumatoid arthritis is a chronic inflammatory disorder that is systemic in nature but is characterized by the manner in which it involves joints. Its onset is usually in the third or fourth decade, although it may begin at any age. Many joints, particularly those of the extremities, are usually afflicted at the same time, often in a symmetric fashion. The course is variable, commonly involving remissions and exacerbations, and the spectrum of clinical manifestations is broad, ranging from barely recognizable forms to debilitating and mutilating ones. To diagnose the disorder, other causes of chronic arthritis should be excluded.

> This process is usually not difficult if the clinical condition is viewed over time. Histopathologic findings are useful but not conclusive, because they overlap with those of other diseases. Rheumatoid arthritis has no specific biologic marker: although rheumatoid factor is present in the serum of most affected individuals, some patients with typical disease are persistently seronegative.
>
> Classic rheumatoid arthritis probably encompasses a heterogeneous group of disorders. At this time, however, it is reasonable to classify as separate disorders only the rheumatoid like diseases that can be directly associated with another major underlying process. (1986, p.1)

Table 1.2 is adapted from the American Rheumatism Association list of criteria for the diagnosis of RA.[43] Five or more of these criteria with joint symptoms lasting in excess of six weeks justify a diagnosis of definite rheumatoid arthritis. A positive result for rheumatoid factor in the absence of other symptoms is not sufficient to yield the diagnosis of definite rheumatoid arthritis.

While rheumatoid arthritis is considered a systemic condition, the criteria presented in the table are concerned almost entirely with the symptoms of the involved joints. As will be discussed later, in greater detail, the treatments for RA provide the most notable effect in the joints as well. The primary reason for investigating means of delivering antirheumatic drugs directly to the joints therefore is to mitigate joint symptoms or alleviate them altogether while lessening the exposure of other parts of

Table 1.2 Criteria and exclusions for diagnosis of rheumatoid arthritis adapted from criteria promulgated by the American Rheumatism Association.[43]

Criteria	Exclusions
Morning stiffness	Rash typical of lupus eryth.
Pain on motion or tenderness in one or more joints	High concentration of lupus eryth. cells in smears
Swelling (soft tissue, not bony overgrowth alone) in one or more joints for more than 6 weeks	Histologic evidence of poly-arteritis nodosa
Symmetric joint swelling with simultaneous involvement	Persistent muscle swelling of dermatomyositis
Subcutaneous nodules in juxta-articular regions	Definite scleroderma (not limited to fingers)
Radiologic changes typical of rheumatoid arthritis	Rheumatic fever with migratory joint involvement
Positive test for rheumatoid factor	Clinical presentation of gouty arthritis
Poor mucin precipitate	Tophi (calcerous crystals)
Characteristic histologic changes in the synovium	Clinical presentation of acute infectious arthritis
Characteristic histologic changes in subcut. nodules	Clinical presentation of a wide variety of arthropathies

the body to systemic toxicity. Consequently, the patient is likely to suffer fewer or less severe side effects. Some agents used for treatment of RA have a maximum cumulative dose. If localized or targeted delivery allows smaller doses to be used, then the patient can be treated longer before the maximum cumulative dose is reached.

1.1.2 Neoplastic Diseases

More popularly known as cancer, neoplastic diseases comprise an ancient and, as yet, poorly understood collection of human afflictions. Neoplasms can be either benign or malignant with the latter known as carcinomas, lymphomas, sarcomas, etc. depending on the stem cell line from which these malignancies derive. These terms (e.g. carcinoma or sarcoma) each cover a multitude of diseases which differ significantly in their clinical presentation, their progression, response to treatment, and even in their reappearance after remission or treatment failure.

Equally diverse are the theories for the origin or origins of cancer.[45] The theories divide along environmental versus genetic lines with the former concentrating on carcinogenic agents. Studies from the 1950s onward linking cancer to cigarette smoking provided the justification for an enormous amount of research into the carcinogenic properties of various cigarette components.[19]

Likewise, research by Peyton Rous at the turn of the century demonstrated a link between cancer and viruses.[45] Yet, after nearly 90 years, conclusive proof is still not to be had. Finally, the genetic aspect of cancer cannot be ruled out either. With so many possible causes, the possible cures must be equally varied.

1.2 Current Treatments

1.2.1 Rheumatoid Arthritis Treatments

Nonsteroidal anti-inflammatory drugs (NSAIDs)have long been the first line treatment for rheumatoid arthritis.[60] Aspirin is the prime example of this class of medications and has long held sway due to its relatively low cost and low toxicity; doses can be as high as 4.8 grams daily.[43] The chief adverse side effects are gastrointestinal in nature, ulceration and subsequent blood loss. At the highest dosage level, aspirin can induce tinnitus resulting in hearing loss.

Hydroxychloroquine and gold salts are the therapies tried first after NSAIDs have been found to be insufficient.[60] These treatments work well but take up to 6 months to begin providing relief. Hence, these are called slow acting anti-rheumatic drugs. Hydroxychloroquine causes severe gastrointestinal upset in approximately 11% of patients while gold salts cause severe mucocutaneous lesions

in 23 to 48% of patients, depending on route of administration.[60] There are other, less serious side effects with these treatments.

The next group of therapies is the cytotoxic drugs. Among these are methotrexate, penicillamine and azathioprine. These drugs can be slow acting, though methotrexate can provide therapeutic results in as little as one month.[60] As the name implies, the primary drawback of this group of drugs is their toxicity. Penicillamine causes mucocutaneous lesions in 30% of patients and nephropathy in 15-20% of patients.[60] Methotrexate and azathioprine are both immunosuppressive and hepatotoxic.

In the treatment of RA, methotrexate is administered weekly either orally in three doses over 24 hours or by a single intramuscular injection.[44] Its hepatotoxicity appears to be cumulative (total dose in excess of 1.5 grams) with an increasing probability of treatment related death occurring after prolonged use, usually more than two years.

Localization of the delivery of methotrexate might be in order given the localization of the primary symptoms. In addition to pain and stiffness, synovial hyperplasia is quite common in rheumatoid joints. Localized delivery of a antifolate would combat this hyperplasia at higher drug concentrations while still making the drug available systemically. It may be possible to achieve the same effect with a significantly lower "whole body" dose of drug. This

in turn would prolong the time needed to reach the 1.5 gram cumulative dose. A goal of this research was to find means of loading protein microspheres to deliver methotrexate in the synovial joint.

1.2.2 Chemotherapy

Surgery is the oldest form of cancer treatment and, often, could only provide temporary relief in the event of metastasis. The oft times systemic nature of the affliction prompted the search for a systemic treatment, thus chemotherapy. Succinctly put, "Cancer chemotherapy is a heterogeneous modality with heterogeneous effects against a heterogeneous group of diseases"(14, p. 65). Systemic chemotherapy suffers from the drawback that, while tumors are often well vascularized, they very often do not absorb toxins well from the blood stream.[35]

From the late 1800s well into this century, a number of antitumor agents were investigated with little success.[45] Not until World War II did researchers isolate drugs with any real potential. The first was the alkylating agent, nitrogen mustard. At nearly the same time, folate antagonists were developed. Methotrexate, discussed above, is perhaps the best known of this class; having some of the earliest demonstrated "cures".[9]

Another important class of drugs is the anthracyclines. Two of the better known examples being doxorubicin

(adriamycin) and daunorubicin. More recently, synthetic anthracene derivatives have been developed, such as mitoxantrone. All of these drugs are effective against a broad range of cancers but have the distinct drawback of being extremely cardiotoxic.[54]

Finally, tumor immunology has produced valuable research in recent years. The possibility that cancerous cells might express antigens not found on normal cells was advanced by Gross in 1943.[45] Gross demonstrated that some level of protection against a challenge with tumor cells could be had from an inoculation made from those same cells in the murine model.

In recent years, research has shown the antitumor activity of certain bacteria derived glycolipids.[59] These endotoxins promote tumor necrosis and regression. A detoxified monophosphoryl lipid A fraction (DMPL) appears to behave as an adjuvant in cancer immunotherapy.[51]

As mentioned before, delivery of these agents locally or even intratumorally would benefit the patient by giving high therapeutic concentrations where needed while minimizing the adverse systemic effects. Localization can be useful pre-operatively as well as post-operatively as a treatment.[48] A goal of this research was, therefore, to examine the synthesis and use of microspheres for the delivery of a chemotherapy agent.

CHAPTER 2
BACKGROUND

2.1 Microspheres

Microparticles have been under investigation as drug carriers for more than three decades.[46] Incorporation of drug into a biologically inert or inactive matrix conveys several advantages for the delivery of that drug and broadens the options available to the clinician.

2.1.1 Applications

There are wide variety of microparticle carriers; ranging from submicron to millimeter diameters. These particles can be uniform throughout (microspheres) or possess an exterior wall or coating that differs significantly from the interior (microcapsules and liposomes). An equally wide variety of manufacturing methods exists. There are several excellent reviews of the development of microparticle carriers and the clinical uses of the same; the reader is referred to Chen *et al.* or Donbrow.[16, 20]

As listed in Table 2.1, there are many reasons for investigating the use of microcarriers for clinical drug

delivery. The earliest uses were for drugs administered orally, where "isolation" can mask unpleasant tastes and protect drugs from stomach acid until the dosage had passed into the intestine. For the purposes of this research, the drugs are far more cytotoxic and possess much more serious side effects.

Table 2.1 Applications of microcarrier technology.

PROBLEM AREA	MICROCARRIER APPLICATION
Storage & Handling	Provide convenient handling and enhance shelf life
Drug Isolation	Isolate drug from digestive juices or body fluids
Release Profile	Control or, more often, sustain release of drug
Drug Instability	Stabilize drugs with short active life spans
Targeting Delivery	Regional localization or actual cell specific targeting of drug

In classical chemotherapy, the systemic use of cytotoxic agents kills both malignant and nonmalignant cells; with the goal being to destroy the tumor without causing the death of the patient. In this situation, the upper limit on the therapeutic dose is that systemic loading that possesses an unacceptable probability of patient mortality.

It is sometimes the case that the drug has organ specific toxicity. One such example is the popular antitumor drug, doxorubicin (adriamycin) which has a unique cardiotoxicity relative to cumulative dose and to peak plasma level.[18] With regional targeting or localization, it is possible to avoid this problem. The drug concentration in the region of implanted microspheres can be at therapeutic levels while the systemic concentration is much lower. By using this delivery method, the therapeutic concentration locally can actually be much higher than the concentration that would kill the patient if such concentration were maintained systemically.

Likewise the release profile is important. Administration of cytotoxic agents, either orally or intravenously, leads to rapid build up in concentration with a fall off that is determined by the metabolism of the drug or its clearance, most often renal clearance, by the body. The organs responsible for the bulk of the metabolism or clearance (e.g. the liver or kidney) may not be the target organ or tissue mass. Controlling the release of the drug at the target tissue mass reduces the burden on nontarget organs.

2.1.2 Design Considerations

The design considerations for the use of microspheres start with the disease or condition to be treated. The disease dictates the drugs that are to be used and the locations to be targeted with those drugs. The location and the drugs chosen place constraints on the types of carrier materials that can be used for making the microspheres. The carrier materials and the drugs used dictate the various other materials used in (as well as all aspects of) the manufacturing process; including but not limited to the formation and stabilization of the spheres, the loading of the drug, the harvesting and packaging of the spheres and drug, and the sterilization of the final product. In selecting the drug, carrier materials and processing agents, one should be mindful of the host response to the final product; e.g. inflammatory response and thrombogenicity.

In addition to material selection, there is the question of physical size and morphology. If the material is to be implanted by injection then, to prevent product settling or clogging of injection cannula, the maximum diameter should be less than 40 μm.[2] If it is desired that the product circulate without clogging capillary structures, then the maximum diameter should be less than 8 μm, though

capillary diameter can be influenced by the use of medications such as vasodilators and vasoconstrictors.[31]

Another morphological consideration is porosity. This will affect the release of drug by diffusion. Porosity will also affect the rate at which the matrix material degrades (if biodegradable) and, thus, affect the rate of release of drugs which are tightly bound to the matrix material.

For the purposes of the research aimed at the treatment of RA, it will be assumed that the microspheres will be used in the knee. As injection is the most likely means of administering the drug; the mean diameter should be less than 40 μm and preferably less than 10 μm. It is preferable that the microspheres biodegrade completely to bioacceptable products at a rate consistent with current treatment schedules for the drug (i.e. methotrexate, discussed subsequently). For this work, that schedule would be approximately one week.

2.2 Choice of Drug

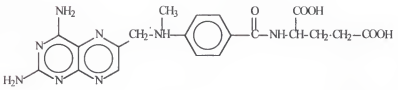
There are a wide variety of compounds that could be studied both for treatment of rheumatoid arthritis and for treatment of neoplastic diseases. Anthracyclines are among the most powerful antitumor agents and do not interfere with other forms of cancer treatment, giving the clinician more

options.[54] A relatively new anthracycline that shows promise is mitoxantrone dihydrochloride. Antimetabolites are another popular group of antitumor agents. Among these is an antifolate, methotrexate, which possesses not only strong antitumor activity but also strong antirheumatoid activity.

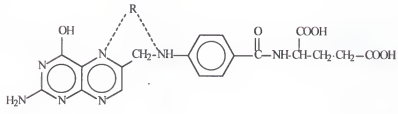
2.2.1 Methotrexate

Methotrexate (MTX), or N-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-glutamic acid, is an anti-cancer/anti-rheumatic drug.[53] The drug is often sold as a sodium salt; this complex forms on the α carboxylic acid moiety of the glutamyl residues. In the non-salt form, MTX is sparingly soluble in water (approximately 1 mg/mL). In solution, MTX has absorption maxima at 244 and 307 nm in acidic media and 257, 302, and 370 nm in basic media.[50] MTX decomposes before melting between 185° and 204° C. It also decomposes in alkaline solutions.

The structure of MTX is shown in Figure 2.1 and bears great similarity to the structure of tetrahydrofolate. This is the key to the mechanism of action for MTX. MTX binds tightly and with great selectivity to the enzyme,



METHOTREXATE



TETRAHYDROFOLATE

Figure 2.1 Chemical structures of methotrexate and of tetrahydrofolate. The high degree of structural similarity is responsible for the antifolate behavior of methotrexate.

dihydrofolate reductase, thereby inhibiting its action.[21] This interrupts two important cellular processes. As depicted in Figure 2.2, rendering dihydrofolate reductase inactive inhibits both thymidylate synthetase and *de novo* purine synthesis. Interruption of the latter process stops production of DNA, RNA, and proteins. Of the two processes, the thymidylate synthetase process appears to be of greater importance to cytotoxicity.[15]

Administering thymidine prior to methotrexate protected normal and malignant cell lines from cell death. It did so by slowing down the rate at which thymidylate synthetase depleted the stock of reduced folates thus preserving the *de novo* purine synthesis process.

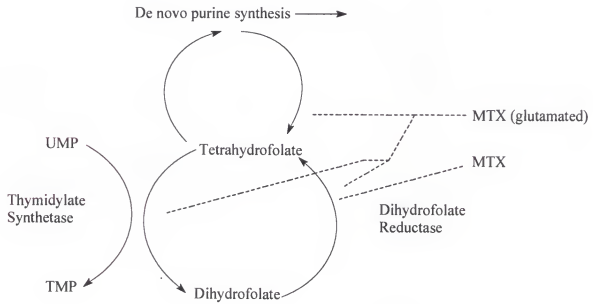


Figure 2.2 Schematic of pathways affected by methotrexate (MTX) and polyglutamated methotrexate. Adapted from Allegra.[1]

For the treatment of RA, MTX is administered as the sodium salt (Na-MTX), either orally or by injection.[34] Typically, the patient is started on 7.5 mg per week (administered in one to three oral doses or injections over

24 hour period) and the dosage is increased, up to 15 mg per week, until the desired results are achieved. Patients are seldom given more than 20 mg per week as there is an increase in the incidence of severe toxic reactions.[44]

There is some disagreement over the fate of MTX or Na-MTX in regard to the synovial joint. In a study of human patients, Tishler *et al.* found following *i.v.* administration of MTX, that serum levels exceeded synovial fluid levels of MTX at two hours.[67] At 24 hours synovial levels exceed serum levels and the drug is undetectable at 72 hours. While Herman *et al.*, in a comparative study using first oral and then repeated with *i.v.* MTX, found "the ratio of synovial fluid concentration to serum concentration . . . to be approximately 1.0" at 4 and 24 hours (30, p. 165). Herman *et al.* found a drug half-life of 6 hours, which is consistent with the finding by Tishler *et al.* of undetectable concentration of the drug at 72 hours.[30, 67]

For this research, the target location is the knee. If efficacy requires systemic doses of 7.5 mg to 15 mg over 24 hours, it is very likely that the amount of drug required for each knee will be significantly less. Certainly, the maximum level needed per knee joint is 7.5 mg of MTX and this is the amount that will be used in calculating the required loading of microspheres.

2.2.2 Mitoxantrone

Mitoxantrone or 1,4 dihydroxy-5,8-bis[(2-[(2-hydroxyethyl)amino]ethyl)amino]-9,10-anthracenedione dihydrochloride is a synthetic anthracene compound approved for the treatment of acute nonlymphocytic leukemia, see Figure 2.3.[22] In the literature, mitoxantrone is often referred to as MTX. The use of both mitoxantrone and MTX would cause confusion in this text; therefore it will be referred to here by a common trade name, Novantrone® or NOV.

NOV is soluble both in ethanol and in water, yielding a dark blue, acidic solution. It decomposes before melting and does so exothermically at 250°C. When administered intravenously (*i.v.*), it is 78% bound to serum proteins.[34] It is incompatible with heparin in that it forms a precipitate and will cause gelation of concentrated solutions of albumin.[22] This result can be explained by carboxylic acid groups on the albumin or heparin complexing with the same sites on NOV as does the hydrochloride or by the acidity of the NOV solution being sufficient to protonate the carboxylic groups, driving the albumin or heparin from solution.

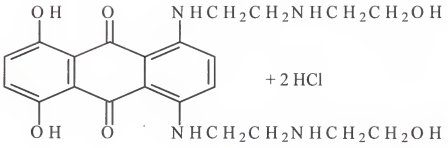


Figure 2.3 Chemical structure of mitoxantrone.

The accepted mechanism for action of NOV is by interaction with DNA.[22] While it is known to intercalate into DNA like other anthracyclines, such as doxorubicin, it does not form oxygen free radicals like these anthracyclines. NOV also forms tight non-covalent bonds with DNA and has been implicated in DNA strand breakages.[34]

The recommended dosage of NOV is 12 mg/square meter of total body surface area. This is done *i.v.* in a minimum of 50 mL of physiologic media (e.g. saline for injection) over 15 to 30 minutes. For the purposes of this research, the microspheres were prepared to saturation with NOV and then were resuspended to achieve the desired concentration.

2.2.3 DMPL

Detoxified monophosphoryl lipid A (DMPL) is a bacteria derived glycolipid which has demonstrated antitumor activity.[59] While lipid A is an endotoxin, the purified

fraction of lipid A shows greatly reduced toxicity and minimal pyrogenicity in animal testing. Takayama et al. demonstrated that while the untreated endotoxin had a $CELD_{50}$ of 0.008 μg in chick embryos, the fraction IV of that same endotoxin had a $CELD_{50}$ (LD_{50}, chick embryo) in excess of 10 μg, or close to four orders of magnitude less toxic.[64] Likewise in a rabbit pyrogenicity test, untreated endotoxin tested positive for pyrogenicity at 0.155 μg while the fraction IV tested positive at 5.0 μg.

To assess antitumor activity, the endotoxin and its fraction IV were compared in a guinea pig hepatocarcinoma model, where untreated animals died in 60 to 90 days post-induction. Treated animals were given 150 μg either of the endotoxin or fraction IV on day 6 post-induction and "cures" were defined as a) complete disappearance of the primary tumor, b) no clinical evidence of metastasis, and c)rejection of contralateral challenge with tumor cells 2 months after initial treatment. Takayama *et al.* report 5 out of 8 (63%) cures for the untreated endotoxin and 7 out of 9 (73%) cures for the fraction IV.[64] It was concluded that the antitumor activity of this endotoxin was not linked to its toxic or pyrogenic activity.

2.2.4 Acid Phosphatase

Acid phosphatases are enzymes found in both animals and plants. These enzymes exhibit catalytic activity toward phosphoesters in an acidic environment.[23] The acid phosphatases can be classified and differentiated by structural properties (i.e. molecular weight and single chain versus dimer); tissue distribution; and common inhibitors (e.g. tartrate resistance).

Acid phosphatases range from molecular weights as low as 18 kDa to in excess of 42 kDa.[4, 23, 70] Those derived from bovine sources exhibit a high degree of homology (85 % to 94 %) with acid phosphatases from human tissues.[23, 70] Acid phosphatase levels in blood have diagnostic value for disorders such as prostatic carcinoma metastasis, thrombocytopenia, and liver disease.

While no therapeutic value is currently attributed to this class of enzymes, there is ongoing interest in the delivery of enzymes by use of microcarriers. Acid phosphatase was chosen for this research for several reasons. It is relatively inexpensive. In comparison to other drugs that might be loaded onto microspheres, it is a relatively large molecule, much closer in size to BSA. Finally, there exist a simple and straightforward colorimetric assay for activity.

The particular enzyme used in this research is derived from bovine milk. It has a molecular weight of 42 kDa and, most likely, is a single chain glycoprotein.[4] The pH optimum for enzyme activity is ca. pH 4.9 and it is inhibited by heavy metals and oxidizing agents.[5]

2.3 Choice of Carrier Materials

Bioacceptability is an important concern in choosing materials for implant construction. An easy means of assuring biological acceptance is to choose materials that are already present in the body. Proteins and polysaccharides that are native to the host organism are unlikely to elicit foreign body or immune responses. There are several tradeoffs to be made in exchange for this benefit.

First, it is often undesirable or unrealistic to rely upon humans as a source for raw materials, for instance the fear of human immunodeficiency virus transmission in harvested tissues. Therefore, the selected materials must have a high degree of trans-species homology. The lower the degree of homology, the greater the possibility of eliciting immune response to the foreign source materials. As well, the harvested materials need to be of high purity to avoid immune response to contaminants. Which leads to the second

set of tradeoffs; the harvesting and purification difficulties make these materials very expensive. Fortunately, in drug delivery situations, the amount of material needed is quite small and, relative to the cost of the drug, often of secondary import.

Finally, natural polymers, such as proteins and polysaccharides, may be difficult to process. Natural polymers are seldom melt processible which leaves some form of solvent processing as the only option. Very often, water is the only solvent available which further constrains processing options.

Since one aspect of this research was directed toward delivering drugs to the synovial capsule, several components of synovial fluid and the concentration of each in both normal and rheumatoid joints were investigated. As can be seen in Table 2.2, the predominant protein in synovial fluid is serum albumin, although its concentration is roughly half that found in blood.[63] The remaining proteins in synovial fluid are globulins.[24] Two important nonprotein components are hyaluronic acid and phospholipids.

Although phospholipid vesicles have been investigated extensively for drug delivery, they were not made a focus of this research. Serum albumin as a microsphere carrier

Table 2.2 Several common components of joint fluid with typical concentrations for normal and rheumatoid joints, adapted from Gatter.[24]

Component	Normal Joint	Rheumatoid Joint
Total Protein	21.0 mg/mL	42.0 mg/mL
Serum Albumin	11.8 mg/mL	17.6 mg/mL
Hyaluronic Acid	3.21 mg/mL	1.15 mg/mL
Phospholipids	0.14 mg/mL	1.01 mg/mL

material has been investigated extensively in this laboratory and, given the high concentration of albumin in synovial fluid, was chosen for this research.[46, 47] Hyaluronic acid was considered important because of its presence in synovial fluid and because it was not a protein. Carboxymethylcellulose, a plant derived polysaccharide which has been demonstrated to work well in certain implantation models, was chosen for study because of its low cost relative to albumin or hyaluronic acid.

2.3.1 Serum Albumin

Serum albumin is the most common of the proteins found in circulating blood.[29] It is comprised of approximately 580 peptide units (species dependent) and has a molecular weight of *ca.* 69,000 Da.[11] It is a water soluble protein with very low solubility in organic solvents.[57] When obtained in commercial quantities, it is most often the

"Fraction V" from the method developed by Cohn.[17] The primary impurities in this fraction are 3-4 % globulins.[57]

The amino acid composition of bovine serum albumin is quite similar to human and a comparison is given in Table 2.3.[29] Still, it must be remembered that even small differences between human and bovine protein can elicit an immune response. The molecule consists of nine sequential loops making up three globular regions, each of similar composition.[11]

Table 2.3 Comparison of amino acid residues in human serum albumin to residues in bovine serum albumin, adapted from Haurowitz, Table VIII-2.[29]

Amino Acid	Human	Bovine	Amino Acid	Human	Bovine
Glycine	14.7	16.7	Alanine	--	48.4
Valine	45.4	34.9	Leucine	62.6	64.6
Isoleucine	9.0	13.7	Proline	30.6	28.5
Phenylala.	32.6	27.5	Tyrosine	17.7	19.3
Tryptophan	0.6	2.0	Serine	24.3	27.8
Threonine	29.0	33.8	Cysteine/ine	36.1	33.9
Methionine	5.93	3.75	Histidine	15.6	17.8
Arginine	24.3	23.4	Aspartic Ac.	53.9	56.5
Lysine	58.0	60.5	Glutamic Ac.	81.6	77.4

This makes for an ellipsoidal molecule with an aspect ratio of roughly 3:1. The radius of gyration for the molecule increases with decreasing pH. Dye binding studies

indicate that the positively charged groups (e.g. lysine) are located on the surface of the hydrated molecule.[29]

With respect to the cross linking of carrier materials to stabilize particulate microcarriers, there are two classes of amino acids which are important. The first class is comprised of amino acids terminated with amine groups. The second class is comprised of amino acids terminated in carboxyl groups. As shown in Table 2.3, the amino acids with amine terminal groups, lysine and arginine, comprise roughly 14% (84/580) of the amino acid residues in bovine serum albumin. While the carboxyl terminated amino acids comprise roughly 23% (134/580) of the amino acid residues on bovine serum albumin.

2.3.2 Hyaluronic Acid

Hyaluronic acid (HA) or β-1,4-glucuronic acid-α-1,3-N acetyl glucosamine is a common extracellular polysaccharide (see Figure 2.4). Commercially it is derived both from animal tissue and from the glycocalyx of common bacteria.

When highly purified, the response it causes *in vivo* is source independent. Solutions of HA derived from bacterial sources have been used as a means of preventing adhesion formation after surgery with no adverse reactions observed.[26] HA is found in acellular locations such as

hyaline cartilage and in the vitreous humor of the eye. It is most often obtained in the form of a sodium salt. HA decomposes before melting and is soluble in water. It is not soluble in common organic solvents.

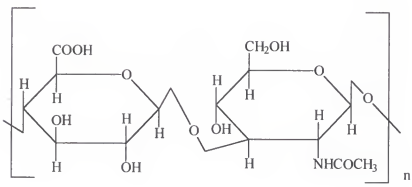


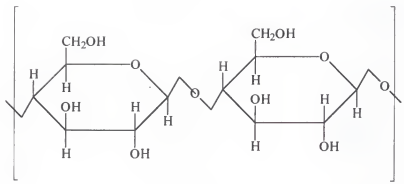
Figure 2.4 Chemical structure of the hyaluronic acid repeat unit.

In nature, this polysaccharide can range from a few thousand to in excess of five million Daltons molecular weight. High molecular weight HA forms extremely viscous solutions at low concentrations (e.g. ca. 400 cps at 0.4% w/v of 2 MDa HA in physiologic saline). With respect to cross linking of this material, the carboxylic group found on the glucuronic acid moiety is the relevant site. This research made use of ionic linkages through this site as a means of cross linking the polysaccharide and stabilizing the microspheres.

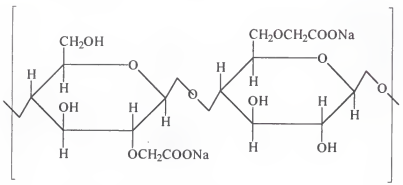
2.3.3 Carboxymethylcellulose

Even HA derived from bacterial sources is a relatively expensive biopolymer. A similar polysaccharide, that is far less expensive, is carboxymethylcellulose (CMC), see Figure 2.5. CMC is a modified, water soluble cellulose derivative. Hydroxyl side groups are carboxymethylated and then converted to the sodium salt. There are three substitution sites per repeat unit and the degree of substitution of a given grade of CMC refers to the average number of sites per repeat unit that have carboxymethyl groups. For example, Aqualon type 7HF CMC has a degree of substitution of 0.65 to 0.90 sites per repeat unit whereas the type 12M8P CMC has a degree of substitution of 1.15 to 1.45 sites per repeat unit.[6, 7]

Like HA, solutions of CMC have been used to prevent adhesion formation in the abdomen, subsequent to surgical manipulation, without adverse reaction.[69] These solutions were compounded from relatively high molecular weight CMC (ca. 700 kDa) so that the desired solution viscosity could be achieved with a minimum amount of CMC. For microsphere compositions, a lower molecular weight is desired so that CMC solution has a reasonable carrier material content (say greater than 5% w/v) and is still of sufficiently low viscosity to be emulsified.



(a)



(b)

Figure 2.5 Chemical structures of a) cellulose and of b) carboxymethylcellulose with a degree of substitution of 1. Note the different locations of substitution.

2.4 Cross Linking

Having chosen serum albumin, hyaluronic acid and carboxymethylcellulose as carrier materials for the microspheres, a means for stabilizing these microspheres was now needed. Depending on the characteristics desired for the final product, microspheres formed during the initial emulsification step must remain separate, stable spheres throughout processing and storage as well as after reconstitution and injection into the patient. This goal is most readily achieved by cross linking the carrier material. Cross links can be comprised of covalent or ionic bonds or even simple "phase separation" of like sections on different chains (as discussed in the appendix).

2.4.1 Constraints and Options

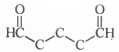
There are several constraints on the choice of cross linking method for the stabilization of microspheres that are to be used *in vivo*. First, any residual cross-linking agent and/or any byproducts from the cross linking reaction must be safe in the body or easily removed in subsequent processing steps. Next, the cross-linking agent must be compatible with the solvents used in processing. For example, there are several interesting cross-linking reactions such as adipyl chloride, that can not be used

because the reaction may require anhydrous conditions and the carrier materials are dissolved in water. Finally, the cross-linking agent cannot compete for the same sites that will be used in complexing the drug to the carrier material. For example, the multivalent metal ion method discussed below is not compatible with the delivery of NOV because these both compete for the carboxyl groups.

2.4.2 Glutaraldehyde

Glutaraldehyde or 1,5 pentanedial is a common cross linking agent for proteins. Aldehydes bind with primary amines via a Schiff base reaction to yield imines, see Figure 2.6.[68] In proteins, the most common binding site by far is to the epsilon amino group on the peptide, lysine although the peptide, arginine, may also play a small role with its terminal diamine.[38, 39] For an excellent review article on glutaraldehyde, the reader is referred to Jayakrishnan and Jameela.[36]

Glutaraldehyde is obtained in aqueous solution in concentrations ranging from a fraction of 1% to 50% w/v. There are several grades of glutaraldehyde ranging from highly purified and frozen scanning electron microscopy grade to commercial grades whose primary contaminants are polyglutaraldehyde.



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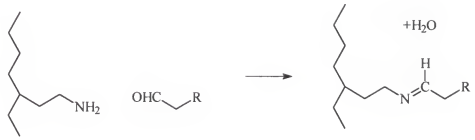

(b)

Figure 2.6 Chemical structure of a) glutaraldehyde and b) schematic of the Schiff base reaction of aldehyde with a primary amine.

Polymerization of dialdehydes has been recognized since the 1930s, though an initial understanding of the mechanisms and structure of polyglutaraldehyde was not available until the 1960s.[52] Thus, glutaraldehyde may exist in a several of forms in aqueous solution including a linear polymeric cyclic semihydrate.[33] The relevance of polyglutaraldehyde to biological implants lies in the cytotoxicity of glutaraldehyde.

Aldehyde related cytotoxicity has been identified in glutaraldehyde treated implants, e.g. heart valve prostheses, and while many have concluded the glutaraldehyde

was freed from such implants by the reversibility of the aldehyde cross links, Huang-Lee *et al.* have concluded that the depolymerization of polyglutaraldehyde contamination of such implants is a more likely source. [33] For this reason, highly purified and frozen S.E.M. grade glutaraldehyde was chosen for this research. It is still possible for polyglutaraldehyde to form during the microsphere synthesis, but the level of contamination should be lower if pure glutaraldehyde is used a component of synthesis than if a commercial grade were used.

There are other dialdehydes which could be tried. One of particular interest is a 3.4 kDa polyethyleneglycol chain terminated with aldehyde groups, see Figure 2.7. This polymer is of interest because the water soluble PEG could serve as a wetting agent. Glutaraldehyde stabilized albumin microspheres tend to be very hydrophobic in the dry state and resuspending such microspheres can be difficult. By including a cross linking agent with a hydrophilic polymer link segment, the resulting microspheres should wet and resuspend more easily. The acetaldehyde is not as stable as the propionaldehyde terminated PEG and so the latter is chosen for this research.[71]

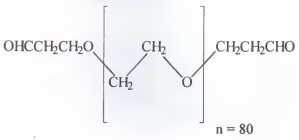

Figure 2.7 Chemical structure of aldehyde terminate polyethyleneglycol.

The maximum amount of glutaraldehyde that can be bound to the carrier material is determined by the number of sites per carrier material molecule. In the case of HA and CMC, there are no sites at which cross links can be established easily using dialdehydes at near neutral pH in aqueous solutions. In the case of bovine serum albumin (BSA) there are (on average) 60.5 lysines per molecule.[29] There are 23.4 arginines, but this peptide has a low binding affinity.

Thus, the maximum amount of glutaraldehyde that could be bound is roughly 60 moles per mole of BSA. This would require every site on a given BSA molecule to be bound, leaving a pendant aldehyde group. If all the sites were bound redundantly, that is to another lysine on the same molecule, the minimum amount of glutaraldehyde is roughly 30 moles of glutaraldehyde. The amount of glutaraldehyde needed saturate all available binding sites ranges from 30

to 60 moles of glutaraldehyde per mole of BSA or, on a mass basis, from 44 mg to 88 mg of glutaraldehyde per gram of BSA. Adding more than this to the reaction process will merely increase the amount that has to be removed during wash processes.

2.4.3 Multivalent Metal Ions

The capture of multivalent metal ions by two or more charged groups on a molecule is known as chelation and has been recognized since early times. Recently the intentional formation of chelates to stabilize polysaccharide solutions has found medical applications. Huang *et al.* have suggested that multivalent metal ions, such as Fe^{3+} or Al^{3+}, can be used to thicken solutions of carboxyl containing polysaccharides, e.g. HA, to make adhesion prevention solutions.[32] While divalent metal ions are theoretically sufficient to provide cross linking, the preferred ions would be trivalent.[32]

All three of the carrier materials selected for this research contain pendent carboxyl groups. Both BSA and HA contain such groups naturally; CMC was derivatized with carboxyl groups for the specific purpose of rendering cellulose water soluble. In each case, the cross linking of the carrier materials would be effected by chelation of the

metal ion by carboxyl groups from two or more chains, see Figure 2.8. While this complex is not stable at all values of pH, in certain pH ranges this linkage stabilizes the molecules.

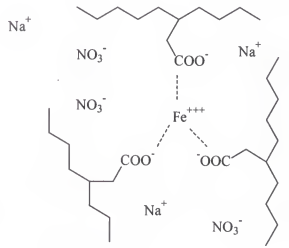

Figure 2.8 Schematic representation of the formation of ionic cross links by the chelation of ferric nitrate by pendent carboxyl groups.

Per molecule, BSA contains 134 peptides (i.e. aspartic acid and glutamic acid) with pendent carboxyl groups. Assuming one ferric ion for every three carboxyl groups, the minimum amount of ferric ion needed to saturate one mole of BSA would 44.67 moles. Using ferric nitrate nonahydrate (for its relatively low toxicity, acetone solubility, and history as a tanning agent for collagen hides) this would translate to 0.262 grams of ferric nitrate ($9H_2O$) per gram of BSA.

CMC, of the variety used in this research, has 0.7 carboxyl groups per repeat group, or 1.428 moles of CMC repeat groups per mole of carboxyl groups. Assuming a minimum of one mole of ferric ion needed per three moles of carboxyl groups to saturate the CMC molecule, this translates to 0.432 grams of ferric nitrate ($9H_2O$) per gram of CMC.

HA has one carboxyl group per repeat group. Again, assuming a minimum of one mole of ferric ion needed per three moles of carboxyl groups to saturate the HA molecule, this translates to 1.066 grams of ferric nitrate ($9H_2O$) per gram of HA.

Adequate stabilization of the microsphere requires that the microsphere matrix be resistant to dissolution in aqueous media for periods ranging from hours to weeks after resuspension and injection into the patient. It may not be necessary to completely saturate the molecule of carrier material with cross links to other carrier molecules in order to achieve effective stabilization. Huang *et al.* suggest that gelation can be achieved with as few as 10% of available carboxyl groups bound up in solutions containing more than 0.5 weight percent of polysaccharide.[32] Still, it is useful to know what will be the upper limits on the amount of cross linking agent needed to stabilize a given

carrier material. Minimizing the amount of ferric ion that is used also minimizes the amount (if any) of unreacted cross linking agent that has to be removed in subsequent processing.

2.4.4 Microwave

Thermal gelation of albumin has been used to stabilize albumin microspheres and involves treating the microspheres between 90 and 180°C. for less than 10 min.[27] This gelation is irreversible and most likely involves rearrangement of cystine-cystine cross links. As many drugs are not heat stable and many solvents used for emulsification boil before 90°C, heating by microwave energy was seen as a possible means of inducing thermal gelation in the individual microspheres without prolonged heating of the emulsion at high temperatures.

2.5 Processing Considerations

Once the materials for the microspheres are chosen, there are still choices to be made as to the selection of certain crucial manufacturing steps and the order of the manufacturing process. Among these choices are the means of stabilizing the emulsion, the means of delivering of the cross linking agent and the means by which the drug is to be introduced to the microsphere.

2.5.1 Surfactant Vs Steric Stabilization

There are two general methods of emulsion stabilization used in this laboratory: a) surfactant in the continuous phase and b) so-called steric hindrance by solutes in the continuous phase. The surfactant method uses a continuous phase comprised of alkanes, e.g. hexane and/or mineral oil and a surfactant such as sorbitan sesquioleate.[37, 40, 41] The surfactant migrates to the interface between the hydrophobic continuous phase and the dispersed aqueous phase (the microspheres) and prevents the agglomeration of the dispersed phase.

The steric hindrance method uses a continuous phase comprised of a solute/hydrophobic solvent system, e.g. polymethylmethacrylate in toluene or cellulose acetatebutyrate in 1,2 dichloroethane (CAB/DCE) in which to disperse the aqueous phase.[46, 47] If the solute is of sufficient molecular weight and is present in sufficient concentration then, by its physical presence, the solute prevents the dispersed phase from agglomerating. One must be careful in choosing the solute. Some polymers, e.g. PVP, are soluble both in water and in DCE. These polymers can cross the interface between the continuous and dispersed phases and lead to massive agglomeration.

Hydrophobicity of the resultant microspheres being an issue of concern, the bulk of this research was performed using the CAB/DCE system. It was feared that residual surfactant on the microspheres was contributing to the hydrophobicity of the product. Since CAB was readily soluble in acetone and extensive acetone rinses are employed to dry the product, the CAB/DCE system seemed least likely to leave significant quantities of emulsion stabilizer behind.

2.5.2 Cross Linking Step

Previous research on protein microspheres performed in this laboratory made use of glutaraldehyde saturated toluene (GST) as a means for delivering the cross linking agent to the emulsion.[40, 46, 47] While this made sense for emulsion systems which contained toluene, it added an extra component to the CAB/DCE system. An extra component means another possible contaminant in the final product. As well, since linear polyglutaraldehyde is comprised of linked cyclic acetals, it was feared that toluene might be a good solvent for this undesirable contaminant.

With DCE was already used in the CAB/DCE emulsion system, it was decided to use it for the delivery of glutaraldehyde. Glutaraldehyde saturated 1,2 dichloroethane

(GSDCE) was compounded in the same way previous workers made GST. Briefly, 25% aqueous glutaraldehyde and DCE were emulsified and left to separate. The preparation was then centrifuged and the top (aqueous) phase removed by pipette.

2.5.3 Pre Vs Post Synthesis Drug Loading

From a processing view point, the simplest means by which to incorporate drug in the microspheres is to make a solution containing both the drug and the carrier material. This is called bulk or *in situ* loading. There are several situations in which bulk loading is not the most desirable means of incorporating the drug.

First, the drug may not be soluble in the same solvent as the carrier material, e.g. water in the case of proteins and polysaccharides. In situations such as this, solid drug can be pulverized to a particle size much smaller than the intended microspheres and added to the solution of carrier material. When emulsification is complete, the microspheres will contain tiny aggregates of the drug dispersed in the carrier matrix.

Second, the drug may be incompatible with the cross linking agent or one of the other processing agents that is chosen. In this case, the drug must be loaded after microsphere stabilization has been achieved.

Finally, it may be desirable to have the drug surface loaded to achieve the release profile that is desired or, in the case of loading antibodies, to make a cell specific, targeted microsphere.

In the research proposed here, MTX has a very low solubility compared to BSA and will be bulk loaded as a fine powder dispersed in the BSA solution. NOV will be loaded after microsphere stabilization because, at the desired drug loading concentrations, NOV would cause the BSA to coagulate.

2.6 Summary of Proposed Research

The hypothesis that treatment of RA would be improved by use of a slow release, MTX delivery system implanted into the synovial capsule requires the development of such a system. This research aimed at demonstrating higher loadings than achieved previously using a protein microsphere model, as well as the effect of drug type, cross linking agent, and extent of cross linking on drug release rates at physiologically relevant conditions

In summary, the work presented here can be broken down into five general sets of goals. First, is the development of a means to deliver MTX or Na-MTX to synovial joints, such as the knee with the aim of getting drug release over 0 to

100 hours. The work will examine loading these two types of methotrexate into BSA microspheres at two loading levels and making use of two different cross link agents and two levels of cross linking.

Second, BSA microspheres loaded with NOV will be produced and examined in two different mouse carcinoma models. Third, the loading of enzyme onto BSA microspheres will be examined both for enzyme release and for activity of retained enzyme. Fourth, microspheres will be produced with various compositions for loading with DMPL. Finally, the production of microspheres using polysaccharides as matrix material and of microspheres using other means of cross linking will be examined.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Unless otherwise specified, materials used for this research were of reagent grade or better. Pharmaceutical agents were used as obtained from the supplier without modification or further purification. Disposable laboratory supplies, e.g. pipette tips and centrifuge tubes, were used whenever possible to minimize the possibility of sample contamination.

3.1.1 Carrier Matrix Materials

The primary carrier matrix material used for these studies was bovine serum albumin (BSA). Unless otherwise specified, the BSA was fraction V by ethanol extraction method (cat.#A-2153, Sigma, St. Louis). The other albumins that were used were lipid-free BSA fraction V (a-BSA, cat.#A-6003, Sigma, St. Louis) and human serum albumin (HSA) made from pooled plasma (cat.#A-1653, Sigma, St. Louis).

BSA is hygroscopic and can contain up to 10% moisture. Moisture content was determined using a "drying balance". Solution concentrations listed in this research will take into account moisture content, i.e. the solution

concentrations are absolute weight by weight or weight by volume. The drying balance automatically heated samples (ranging from 0.5 to 1.5 g) to 130°C over a period of 30 minutes and presented the decrease in mass as well as the percent moisture content. These samples were not used in research because the effect of heating on some the component materials was unknown.

Poly-L-glutamic acid (PGA) was added to serum albumin microspheres in certain lots to increase the carboxyl groups concentration. In this research, the 39 kDa sodium salt of PGA was used (cat.#P-4886, Sigma, St. Louis)

HA was obtained courtesy of Genzyme Corp. in the form of a 0.4% w/v solution (Sepracoat[tm] Coating Solution, Genzyme Corp., Cambridge, MA). CMC was obtained as a sodium salt courtesy of Aqualon in the form of a dry powder (moisture content varied and was accounted in a manner similar to BSA). The type used was a low molecular weight CMC with a degree of substitution of approximately 0.7 out of 3 (type 99-7L2P, Aqualon, Wilmington, DE).

3.1.2 Cross Linking Agents

The glutaraldehyde used in this research was S.E.M. grade, 25% aqueous solution that is highly purified and shipped frozen in 10 mL aliquots (cat.#G-5882, Sigma, St. Louis, MO). Prior to use in this research, this aqueous glutaraldehyde was transformed into glutaraldehyde saturated

1,2 dichloroethane (GSDCE). Briefly, this involved making an emulsion of the approximately 10 mL of aqueous glutaraldehyde in approximately 30 mL of DCE in a 50 mL conical centrifuge tube. The tube was tared and each component weighed after addition. After emulsifying, the tube was left on a rotary sample tumbler from 12 to 24 hours. The tube was then centrifuged (5 minutes at ca. 1,800 g's). The aqueous (top) phase was scrupulously removed by pipette and the tube weighed again. The difference in mass between the final weighing and the initial DCE added was presumed to be glutaraldehyde.

The glutaraldehyde mass per mL was calculated for each batch of GSDCE made. These ranged between 32.5 mg/mL and 53.8 mg/mL with a mean of 41.0 $\pm$ 7.7 mg/mL ($\pm$ sample standard deviation, n = 6). During microsphere synthesis, the amount of GSDCE added was adjusted to compensate for the actual concentration of the batch of GSDCE being used.

The aldehyde modified PEG (a-PEG) used was obtained as a fine, dry white powder and stored in the freezer until use (cat.#ALD-3400, Shearwater Polymers, Inc., Huntsville, AL). This material was a 3.4 kDa linear PEG terminated with propionaldehyde on each end. Moisture content was unknown. This material is extremely heat sensitive and was obtained only in small quantity. The moisture content analysis could not be performed on this material as the heating involved would destroy the entire sample. So, for the purposes of

this research, the moisture content was presumed to be zero for any calculations relating to cross linker or drug concentration.

The multivalent metal ion chosen for this research was the ferric ion. The reagent used was either ferric sulfate anhydrous (Mallinckrodt, St. Louis, MO) or ferric nitrate nonahydrate (cat.#F8508, Sigma, St. Louis, MO).

3.1.3 Drugs

Methotrexate (MTX) was obtained courtesy of Lederle Laboratories as a fine dry powder. Sodium methotrexate (Na-MTX) was purchased as a lyophilized powder (NDC 0205-4653-02, Lederle Parenterals, Inc., Carolina, Puerto Rico). Mitoxantrone (NOV) was obtained as a dry powder of 82% purity, courtesy of Lederle Laboratories. Loading calculations for NOV will take the percent purity into account.

3.1.4 Equipment, Materials and Supplies

All aqueous solutions were made using ultra filtered, deionized water obtained from a Nanopure[tm] water purification system (model #D4754, Barnstead/Thermolyne, Dubuque, Iowa) which produced water of ca. 18 megaohm quality. This ultra filtered, deionized water used for solution preparation will be referred to as "ultrapure water" to distinguish it from the tap water used for non-critical laboratory processes, e.g. water baths.

Both the initial emulsification and subsequent resuspensions were done by vortexing (Vortex Genie 2, Scientific Ind., Inc., Bohemia, NY). Centrifugations were performed on a bench top centrifuge (Dynac II, Clay Adams, USA) with a four place rotor of 14.0 cm effective radius. This research made use of rotor speeds between 2,800 and 3,500 rpm or centripetal accelerations of between ca. 1,200 and ca. 1,900 g's.

Weighing and moisture determinations were performed on moisture analyzing digital balance (model LJ16, Mettler Inst. Group, Highstown, NJ). Measurements to a fraction of a mg were performed on a model A-200DS digital balance (Denver Inst. Co., USA) fitted with a wind screen.

Spectrophotometric measurements were performed on either a model Lambda 3B (Perkin-Elmer, Oak Brook, Illinois) or a Superscan 3 (Varian Techtron Ltd., Springvale, Australia). Statistical analysis was done using GraphPad Instat Mac (GraphPad Software, San Diego, CA) on a Macintosh SE (Apple Computer, Cupertino, CA) or with a hand held calculator (model HP-11C, Hewlett-Packard, Corvallis, Oregon).

3.2 Methods: Microsphere Synthesis

All microsphere syntheses for this research were variations on stabilized emulsion techniques developed by

Goldberg and Longo. To facilitate tracking, the various batches of microspheres will be referred to by their respective lot numbers.

Two common measurements performed during synthesis were mean diameter determination and overall yield. Mean diameter measurements were done after the cross linking step with an optical microscope (Nikon Fluophot, Nikon, Japan) using a 100 Xobjective lens (Plan 100, Nikon, Japan) and a 10 X, reticule eyepiece (CFW 10X, Nikon, Japan). Values reported are the average of 100 microspheres plus and minus one sample standard deviation.

The overall yield was calculated as a percent of theoretical maximum yield by dividing the actual yield mass by sum of the masses of the components (i.e. initial carrier mass plus initial drug mass plus cross linker mass). Given the small quantities being manipulated, no attempt was made to determine final moisture content, as the moisture determination process would use up all of the product. In cases where little product was lost in manufacturing and the drying was incomplete, it was possible to calculate overall yields greater than 100%.

3.2.1 Methotrexate Studies

A total of eleven lots of microspheres were prepared using glutaraldehyde as the cross linking agent, see Table 3.1. All lots, except 9618, were produced using the same

processing procedure and the compositions listed in the table. Lot 9618 was produced with no glutaraldehyde. This lot was produced to validate the need for the cross linking agent in microsphere stabilization, and otherwise was processed the same as the other lots.

For each lot, approximately 2.5 g of 20.0% w/w aqueous BSA solution was placed into a tared 50 mL conical bottom centrifuge tube with a measured amount of either no drug, MTX, or Na-MTX (see Table 3.1 for actual amounts for each lot). To each tube, 16.0 mL of 2% w/v CAB/DCE solution was added. The tubes were vortexed for 240 seconds on the highest setting. At this time, either 0.30 or 0.60 mL of GSDCE was added to each tube and the tube was immediately vortexed for another 60 seconds. The tubes were placed on a rotary sample tumbler for two hours.

Then each tube was made up to 50 mL by adding acetone (i.e. approximately 31 mL of acetone was added for a resultant suspension volume of ca. 50 mL) and agitated briefly by hand. The tubes were spun down for 5 minutes at 3,400 rpm to yield clear supernatant over tight pellets. The supernatant was decanted and the acetone rinse repeated twice (made up to 50 mL each time).

At this time, 5.0 mL of 1.0% w/w BSA solution (aqueous) was added to cap any pendent aldehyde groups. The pellets were resuspended and the tubes placed on the rotary sample tumbler for one hour. The tubes were spun down again (5

min. at 3.,400 rpm) and the supernatant was scrupulously removed with a pipette and saved for later analysis.

Table 3.1 Compositions for the MS lots produced for the MTX studies using glutaraldehyde to cross link the BSA matrix. All masses in grams.

Lot #	Amount of BSA	Amount of Glutarald.	Drug type	Amount of Drug
9618	0.507	0	N/A	0
9619	0.499	0.010	N/A	0
9620	0.501	0.020	N/A	0
9621	0.509	0.010	MTX	0.253
9622	0.489	0.020	MTX	0.246
9623	0.492	0.010	MTX	0.491
9624	0.494	0.020	MTX	0.490
9628	0.494	0.010	Na-MTX	0.246
9629	0.504	0.020	Na-MTX	0.250
9630	0.496	0.010	Na-MTX	0.493
9631	0.501	0.020	Na-MTX	0.505

The tubes were made up to 50 mL by adding acetone and the pellets resuspended, either by vortexing or by agitating by hand. The tubes were spun down (5 min. at 3,400 rpm) and the supernatant decanted. The acetone drying rinses were repeated twice (made up to 50 mL each time).

The tubes were covered with filter paper and left to air dry overnight in the hood. The tube tare weight was subtracted from the final weight to calculate lot yield.

The tubes were sealed and stored at ambient temperature until used in the drug and protein elution assays.

There were a total of ten lots prepared using ferric nitrate nonahydrate as the cross linking agent, see Table 3.2. All lots were produced by the same processing procedure which was a variation on the one given above.

For each lot, approximately 2.5 g of 20.0% w/w aqueous BSA solution was placed into a tared 50 mL conical bottom centrifuge tube (see Table 3.2 for actual amounts for each lot). To each tube, 16.0 mL of 2% w/v CAB/DCE solution was added. The tubes were vortexed for 240 seconds on the highest setting. At this time, 5.0 mL of either 13 mg/mL or 26 mg/mL ferric nitrate nonahydrate in acetone was added to each tube and the tube was immediately vortexed for another 60 seconds. The tubes were placed on a rotary sample tumbler overnight.

Then each tube was made up to 50 mL by adding acetone, agitated briefly by hand, and placed back on the rotary sample tumbler overnight. Then the tubes were spun down for 5 minutes at 3,400 rpm to yield slightly tan supernatant over tight pellets. The supernatant was decanted and the acetone rinse repeated twice (made up to 50 mL each time).

At this time, 5.0 mL of 1% w/w aqueous BSA solution was added to cap any pendent aldehyde groups. The pellets were resuspended and the tubes placed on the rotary sample tumbler for one hour. The tubes were spun down again (5

min. at 3.,400 rpm) to yield milky yellow supernatants. The high ferric content lots (e.g. 9702, 9704, etc.) gave less supernatant and had a more milky appearance. The supernatant was scrupulously removed with a pipette and saved for later analysis.

Table 3.2 Compositions for the MS lots produced for the MTX studies using ferric nitrate nonahydrate to cross link the BSA matrix. All masses in grams.

Lot #	Amount of BSA	Amount of $Fe(NO_3)_3$	Drug type	Amount of Drug
9637	0.501	0.130	N/A	0
9638	0.502	0.065	N/A	0
9701	0.505	0.065	MTX	0.248
9702	0.507	0.130	MTX	0.248
9703	0.502	0.065	MTX	0.499
9704	0.503	0.130	MTX	0.501
9705	0.501	0.065	Na-MTX	0.248
9706	0.500	0.130	Na-MTX	0.249
9707	0.500	0.065	Na-MTX	0.499
9708	0.505	0.130	Na-MTX	0.498

The tubes were made up to 50 mL by adding acetone and the pellets resuspended, either by vortexing or by agitating by hand. The tubes were spun down (5 min. at 3,400 rpm) and the supernatant decanted. The acetone drying rinses were repeated twice (made up to 30 mL each time).

The tubes were covered with filter paper and left to air dry overnight in the hood. The tube tare weight was subtracted from the final weight to calculate lot yield. The tubes were sealed and stored at room temperature until used in assays.

3.2.2 Novantrone[tm] Studies

A total of three NOV loaded lots plus an unloaded control were prepared. Lot 9201 was produced in four batches that were combined near the end of processing. This lot was used in the LLC studies described in section 3.4.1. Lots 9306, 9307, and 9308 were produced for use in the MOT studies described in section 3.4.2, see Table 3.3.

Lot 9201 was prepared by the method of Knepp in four batches. For each batch, 40 mL hexane, 10 mL of light mineral oil, and 1.0 mL of sorbitan sesquioleate (Arlacel[tm] 83, ICI Americas, Wilmington, DE), was placed in a 250 mL Florentine flask. To this was added 2.0 mL of 20% w/v BSA, 5% w/v PGA solution (aqueous). Teflon paddles were inserted into the flask and the contents mixed for 15 minutes at 5,000 rpm (Carfamo mixer). At this time, 10 mL of glutaraldehyde saturated toluene was added (glutaraldehyde concentration unknown) and the mixture was left to mix 24 hours at low (<<1000 rpm) speed at room temperature.

Table 3.3 Compositions for the MS lots produced for the Novantrone[tm] studies. All masses in grams.

Lot #	Matrix Material	Amount	Cross Linker	Amount	Capping Material
9201*	BSA PGA	2.0 0.5	glutarald. (toluene)	---	---
9306	BSA PGA	0.410 0.101	glutarald. (DCE)	0.037	BSA
9307	BSA PGA	0.410 0.101	glutarald. (DCE)	0.037	BSA
9308	BSA PGA	0.410 0.101	glutarald. (DCE)	0.037	BSA

* prepared in four batches

The mixture was transferred to centrifuge tubes, spun down for 5 minutes at 2,000 rpm, and decanted. The tubes were made up to 15 mL by adding acetone, resuspended, and centrifuged again (5 min. at 2,000 rpm). The acetone rinses were repeated five times, with the pellets combined between rinse two and three. The batches were dried in vacuum (ca. 29 in. Hg, room temperature) for 30 minutes. The batches were combined when dry.

The MS were loaded with NOV by placing 0.500 g of lot 9201 in a 50 mL conical bottom tube and adding 4.9 mL of aqueous NOV solution (34.7 mg/mL). The tube was vortexed to resuspend the MS and then placed on rotary sample tumbler for 23 hours. This was centrifuged (5 min. at 2,700 rpm) to yield near clear supernatant over a tight blue pellet. A total of 2.2 mL of supernatant was pipetted off and saved

for later analysis. Then, 10.0 mL of acetone was added to the tube and the pellet suspended by vortexing. This was spun down (5 min. at 2,700 rpm) and 10.0 mL of deep transparent blue supernatant removed for later analysis. The rinse was repeated and 9.5 mL of light blue supernatant retrieved.

The tube was covered with filter paper and dried in vacuum as before. The tube was sealed and stored at room temperature until use.

Lots 9306, 9307, and 9308 were produced using CAB/DCE as the continuous phase. For each lot, 16.0 mL of 2% w/v CAB/DCE was placed in a 20 mm x 150 mm screw top test tube. To this was added 2.2 mL of 20%/5% w/v BSA/PGA in aqueous solution. The tubes were vortexed for 120 seconds at the highest setting, then 1.0 mL of GSDCE (37.2 mg/mL) was added, and the vortexing continued for another 12 seconds. The tubes were then placed on the rotary sample tumbler for 20 hours.

The lots were then transferred to tared 50 mL conical bottom tubes and made up to 50 mL by adding acetone. The tubes were centrifuged for 15 minutes at 3,400 rpm to yield clear supernatant over tight pellets. The rinse was repeated three times.

At this time, lots 9306 and 9307 got 15 mL of 2% w/w aqueous BSA solution to cap pendent aldehydes. Lot 9308 received 5.15 g of 3.4% w/w aqueous NOV solution. All lots

were resuspended and the tubes placed on the rotary sample tumbler overnight at ambient temperature.

The tubes were spun down (15 min. at 3,400 rpm) to yield clear supernatants for lots 9306 and 9307 and dark blue supernatant for lot 9308. The supernatants were pipetted off and saved for later analysis. The tubes were made back up to 45 mL in ultrapure water and centrifuged again (60 min. at 3,400 rpm). The supernatants were decanted and saved.

At this time, lot 9306 received 5 mL of ultrapure water; lot 9307 received 4.98 g of 3.4% w/w aqueous NOV solution; and lot 9308 received 14 mL of 2% w/w aqueous BSA solution to cap any pendent aldehyde groups. The tubes were vortexed and placed on the rotary sample tumbler overnight.

The tubes were spun down (20 min. at 3,400 rpm) and the supernatants decanted and saved. The tubes were made up to 50 mL by adding acetone and resuspended by vortexing. The tubes were spun down (15 min. at 3,400 rpm) and the supernatants saved. The acetone drying rinses were repeated twice more.

The tubes were covered with filter paper and dried in vacuum as before for 90 minutes. The tubes were sealed and stored at room temperature until use.

3.2.3 Acid Phosphatase Studies

This study investigated the loading of acid phosphatase into (*in situ* loading) and onto (post-loading) BSA microspheres. Acid phosphatase (type V, from bovine milk, cat.#P-1267, Sigma, St. Louis) was obtained as a dry powder containing 26% to 35% protein with the balance being buffer salts such as sodium citrate.

Two lots of post-loaded MS were produced. The first lot, 9614 was produced from lot 9613 (see Table 3.4). Lot 9613 was produced by dissolving 0.528 grams of BSA in 1.926 grams ultrapure water (20.0% w/w) in a 50 mL conical tube. To this was added 16.0 mL of 2.0% w/v CAB/DCE. The BSA solution was emulsified by vortexing on highest setting for 240 seconds. To this was added 0.5 mL of GSDCE (39.1 mg glut./mL) and this was vortexed for another 60 seconds. The emulsion was agitated gently on a rotary tumbler for 80 minutes at room temperature.

The emulsion was made up to 50 mL by the addition of acetone and agitated briefly by hand. This was spun down for 5 minutes at 3,000 rpm to yield clear supernatant over a tight pellet. The supernatant was decanted and the pellet resuspended in 50 mL of fresh acetone and centrifuged again (5 min. at 3,000 rpm). Again the clear supernatant was decanted and the acetone rinse repeated one more time.

After decanting, 5.0 mL of 1% w/w aqueous BSA solution was added and the pellet resuspended; this was done to cap any free aldehyde groups with BSA. The sample was returned to the rotary tumbler for one hour. The tube was spun down (5 min. at 3,000 rpm) and the clear supernatant decanted. The suspension volume was made up to 50 mL by adding acetone and agitated briefly by hand. The tube was spun down (5 min. at 3,000 rpm) to yield clear supernatant over a tight pellet; the supernatant was discarded. This rinse procedure was repeated three more times and then the pellet was left covered with filter paper to air dry overnight.

Final product weight was 0.489 grams. From this, 0.220 grams of MS were added to 87 mg of acid phosphatase in 5.5 mL of ultrapure water in a 15 mL conical tube to produce lot 9614 (see Table 3.4). The MS resuspended with some difficulty. This suspension was allowed to sit refrigerated for 100 minutes. The tube was spun down (5 min. at 3,000 rpm) to yield 4.5 mL of clear supernatant. The supernatant was removed and the pellet resuspended in 15 mL of acetone. This was spun down (5 min. at 3,000 rpm) and the supernatant decanted. This rinse step was repeated and the pellet was covered with filter paper and allowed to air dry overnight. The final weight was 0.183 grams (assuming the buffer salts were lost in processing, a 73% yield). No assay was run to determine uptake of enzyme, so for the purpose of the subsequent elution study, the loading was assumed to be

equivalent to 87 mg of acid phosphatase divided by 307 mg (the starting mass of MS plus enzyme) or 284 µg acid phosphatase per mg total MS mass.

The second post-loaded lot, 9616, was produced similar to lot 9614 with the following exceptions (see Table 3.4). The initial solution used 0.526 grams of BSA in 1.987 grams of ultrapure water (20.1% w/w). The suspension was allowed to cross link for two hours. A solution of acid phosphatase (4.8 mL of a 25 mg in 5.0 mL ultrapure water solution) was used in place of the BSA capping solution. Three 50 mL acetone rinses with centrifugation were used prior to air drying. The final weight was 0.568 grams (99% yield, this high value most likely due to incomplete drying). The enzyme activity assay (described subsequently in the section on acid phosphatase elution studies) was run on aliquots of the enzyme solution both before and after treatment of the MS. The results of that assay indicate that 92% was taken up by the MS for a loading density of 38.6 µg of acid phosphatase per mg of total MS mass.

The *in situ* loaded lot, 9617, was produced at the same time at lot 9616. The initial solution contained 0.520 grams of BSA and 0.045 grams of acid phosphatase. When 9616 was capped with the enzyme, lot 9617 was capped with 4.8 mL of 1% aqueous BSA solution and was recovered using three 50 mL acetone rinses prior to air drying overnight. The final

weight was 0.606 grams (104% yield, most likely due to incomplete drying). Assuming no loss of enzyme during processing, the loading density was 45 mg divided by 606 mg or 74.3 µg acid phosphatase per mg of total MS mass.

Table 3.4 Compositional variations in microsphere lots produced for the acid phosphatase studies. All masses in grams.

Lot #	Mass of BSA	Mass of Acid Phos.	Post Load or *In Situ*	Mass of Glut.
9613	0.528	N/A	N/A	0.020
9614	0.220 (of 9613)	0.087	Post	N/A
9616	0.526	0.024	Post	0.020
9617	0.520	0.045	*in situ*	0.020

3.2.4 DMPL Studies

Eight lots of microspheres of various compositions were prepared for use by Dr. John Cantrell of Ribi ImmunoChem Research, Inc. (Ribi). All lots were prepared by a similar protocol with variations in the carrier matrix materials and cross linking agent that were used.

Three lots (9313, 9316, and 9317) of MS were produced using 20 mm x 150 mm, screw top test tubes. Each of these lots was produced by dissolving the masses of BSA and PGA (see Table 3.5) in sufficient ultrapure water to yield a solution that was 19.0% w/w BSA in the test tube. To this

was added 16.0 mL of 2.0% w/v CAB/DCE. The carrier solution was emulsified by vortexing on highest setting for 120 seconds. To each tube was added 1.0 mL of GSDCE (37.2 mg glut./mL) and this was vortexed for another 60 seconds. The emulsions were agitated gently on a rotary tumbler overnight at room temperature.

The emulsions were made up to 50 mL by the addition of acetone and agitated briefly by hand and returned to the tumbler overnight. Then these were spun down for 15 minutes at 1,900 g's to yield clear supernatant over a tight pellet. The supernatant was decanted and the pellet resuspended in 50 mL of fresh acetone and centrifuged again (15 min. at 1,900 g's). Again the clear supernatant was decanted and the acetone rinse repeated two more times.

After decanting, 10.0 mL of 1% w/w aqueous BSA or a-BSA solution was added to each tube (see Table 3.5) and the pellet resuspended; this was done to cap any free aldehyde groups with serum albumin. The samples were returned to the rotary tumbler overnight at room temperature. The tubes were spun down (15 min. at 1,900 g's) and the clear supernatant decanted. The suspension volume was made up to 40 mL with ultrapure water and agitated briefly by hand. The tubes were spun down (15 min. at 1,900 g's) to yield clear supernatant over a tight pellet; the supernatant was discarded.

The tubes were made up to 50 mL by adding acetone and resuspended by vortexing. This was centrifuged (15 min. at 1,900 g's) This rinse procedure was repeated one more time and the tubes were left covered with filter paper to air dry overnight.

Table 3.5 Compositions and amounts for the lots produced for use by Ribi ImmunoChem in the DMPL studies. All masses in grams.

Lot #	Matrix Materials	Amount	Cross Linker	Amount	Capping Material
9313	BSA	0.460	glutarald.	0.037	a-BSA
9316	BSA PGA	0.323 0.080	glutarald.	0.037	BSA
9317	BSA PGA	0.323 0.080	glutarald.	0.037	a-BSA
9318	BSA	0.503	$Fe(NO_3)_3$	0.159	BSA
9319	BSA PGA	0.400 0.100	glutarald.	0.037	BSA
9320	a-BSA PGA	0.400 0.100	glutarald.	0.037	a-BSA
9404*	BSA PGA	1.607 0.404	glutarald.	0.155	BSA
9405	HSA PGA	0.412 0.103	glutarald.	0.039	HSA

*produced in four batches

Lots 9318, 9319, and 9320 were produced by a similar protocol with the following exceptions. The types and amounts of carrier matrix materials are listed in Table 3.5. The ferric nitrate was dissolved in 20 mL of acetone before

it was added to lot 9318. Conical bottom, 50 mL centrifuge tubes were used in place of test tubes. The post-capping water rinse was omitted and there were four acetone drying rinses.

Lots 9404 and 9405 were prepared by a similar protocol with the following exception. The types and amounts of carrier matrix materials is listed in Table 3.5. Conical bottom, 50 mL centrifuge tubes were used in place of test tubes. Lot 9404 was produced in four batches. Initial emulsion vortexing was 180 seconds followed by addition of cross linking agent and another 60 seconds of vortexing. GSDCE concentration was 53.8 mg of glutaraldehyde per mL. The post-capping water rinse was omitted and there were three acetone drying rinses.

Loading of DMPL onto microspheres was performed post synthesis. This work was done at Ribi by Dr. John Cantrell. Briefly, the DMPL was dissolved in either ethanol or in triethylamine. The solution was added to the microspheres and incubated for a set time. Following incubation, the MS were spun down; the supernatant was decanted (and saved for testing), and the MS were rinsed in water and centrifuged three times (each rinse was also saved). The microspheres were then dried.

3.2.5 Miscellaneous MS Synthesis Studies

This section contains the MS synthesis methods for four small studies done on: 1) HA as a carrier matrix material (including a BSA/HA mix); 2) CMC as a carrier matrix material; 3) propionaldehyde terminated PEG (a-PEG) as a cross linking agent; and 4) microwave exposure as a cross linking agent for BSA MS. Table 3.6 presents the lot designations and the compositions.

Table 3.6 Lot designations and compositions for studies on HA and CMC as matrix materials and on a-PEG and microwave exposure as cross linking agents for stabilizing BSA MS. All masses in grams.

Lot #	Matrix Material	Amount	Cross Linker	Amount	Capping Material
HA-1	HA	0.040	$Fe_2(SO_4)_3$	0.005	N/A
HA-2	HA	0.040	$Fe_2(SO_4)_3$	0.010	N/A
HA-3	HA	0.040	$Fe_2(SO_4)_3$	0.010	N/A
9641	BSA HA	0.510 0.080	glutarald.	0.020	BSA
9639	CMC	0.050	$Fe(NO_3)_3$	0.130	BSA
9640	CMC	0.050	$Fe(NO_3)_3$	0.065	BSA
9635	BSA	0.151	a-PEG	0.101	BSA
9636	BSA	0.101	a-PEG	0.150	BSA
9709	BSA	0.502	microwave	30 sec.*	BSA

* 900 watt field at 2450 MHZ

3.2.5.1 HA carrier matrix material

Three attempts were made to produce microspheres using HA as the carrier matrix, see Table 3.6. Lot HA-1 made use of 40.0 mL of 1.0% CAB/DCE as the continuous phase and 1.0 mL of 0.4% w/v HA solution. This was emulsified by over 60 seconds of sonication (power level 6, model W375, Heat Systems-Ultrasonics, Inc., Stoughton, MA). At this point, 1.0 mL of 0.5% w/v aqueous ferric sulfate solution was added and sonication continued for another 60 seconds. This was left on the rotary sample tumbler overnight.

After centrifugation (3 min. at 2,800 rpm) the supernatant was decanted from under a tight floating pellet (DCE has density greater than 1.0 g/cc). When acetone was added to dry the microspheres, the pellet dissolved into shreds.

Lots HA-2 and HA-3 were variations on the method of Knepp using hexane or light mineral oil, but no surfactant, as the continuous phase. Briefly, 2.0 mL of 0.4% w/v HA solution was dispersed in 40 mL of alkane by sonication for 60 seconds. To this was added 2.0 mL of 0.5% aqueous ferric sulfate and sonication was continued for another 60 seconds.

As expected, hexane provided a less stable emulsion than light mineral oil. Upon centrifugation (4 min. at 2,800 rpm), the water phases in each batch contained stable

microspheres. However, the addition of acetone to dry the microspheres caused these to rapidly shrivel up. This was to be expected, given that the starting solution was only 0.4% HA. Even if a stable gel had been formed by the addition of ferric ion, this gel was highly swollen with water and should collapse with exposure to acetone.

Finally, a mixture of BSA and HA was tried. Lot 9641 made use of CAB/DCE as the continuous phase. Dry BSA was added to 2.0 mL of 0.4% w/v HA solution in a 50 mL conical bottom tube, see Table 3.6, and when dissolved, this was emulsified by adding 16.0 mL of 2.0% w/v CAB/DCE and vortexing for 240 seconds on the highest setting. To this was added 0.6 mL of GSDCE (32.5 mg/mL) and the vortexing continued for another 60 seconds. This was placed on the rotary sample tumbler for two hours.

The tube was made up to 50 mL by adding acetone and agitated briefly by hand. This was centrifuged (5 min. at 3,400 rpm) and the supernatant decanted. The tube was made back up with 30 mL of acetone and the pellet resuspended. This was centrifuged again and, after decanting, 5.0 mL of 1% aqueous BSA solution was added. The pellet was resuspended and placed on the rotary sample tumbler for three hours.

The tube was then spun down and the supernatant decanted. The tube was made up to 50 mL by adding acetone and the pellet resuspended. This was centrifuged (5 min. at 3,400 rpm) and the supernatant decanted. The acetone rinses were repeated two more times and the tube covered with filter paper and left at room temperature to dry overnight, drying was done in a hood to ventilate acetone fumes . Protein elution was measured as described in section 3.3.2.

3.2.5.2 CMC carrier matrix material

Two lots using CMC as the carrier matrix material were prepared, see Table 3.6. Lots 9639 and 9640 were produced in an identical manner with the exception of how much cross linking agent was used. For each lot, a 2.0% w/w aqueous solution of CMC (99-7L2P, Aqualon, Wilminton, DE) was added to a 50 mL conical bottom tube with 16.0 mL of 2.0% w/v CAB/DCE. This was emulsified by vortexing for 240 seconds on the highest setting. To each emulsion was added 5.0 mL containing ferric nitrate nonahydrate, see Table 3.6 and the vortexing continued for another 60 seconds. At this point, some curds formed in the otherwise milky or milky/rust colored emulsions. The tubes were placed on the rotary sample tumbler overnight.

The tubes were made up to 50 mL by adding acetone and agitated briefly by hand. These were spun down (5 min. at 3,400 rpm) and the slightly tan supernatants decanted. The acetone rinses were repeated twice. To each tube was added 5.0 mL of 1% aqueous BSA solution and the pellets resuspended easily. The tubes were put on the rotary sample tumbler for two hours and then spun down (5 min. at 3,400 rpm). The supernatants were decanted, the tubes made back up to 50 mL by adding acetone, and the pellets resuspended. The tubes were spun down (5 min. at 3,400 rpm) and decanted. The acetone rinses were repeated twice more. The tubes covered with filter paper and left to dry in the hood for two days.

3.2.5.3 a-PEG cross linking study

Two lots using BSA as the carrier matrix material and a-PEG as the cross linker were prepared, see Table 3.6. Lots 9635 and 9636 were produced in an identical manner with the exception of how much cross linking agent was used. For each lot, a 20.1% w/w aqueous BSA solution was added 20.1% w/w aqueous a-PEG solution, see Table 3.6, in a 50 mL conical bottom tube. To each was added 16.0 mL of 2.0% w/v CAB/DCE. This was emulsified by vortexing for 240 seconds on the highest setting. At this point, the tubes were placed on the rotary sample tumbler overnight. The tubes

then were made up to 50 mL by adding acetone and returned to the rotary sample tumbler.

Then the tubes were spun down (5 min. at 3,400 rpm) and the supernatants decanted. The acetone rinses were repeated once. To each tube was added 5.0 mL of 1% aqueous BSA solution and the pellets resuspended easily. The tubes were put on the rotary sample tumbler for one hour and then spun down (5 min. at 3,400 rpm) to yield tan solutions with no microspheres.

3.2.5.4 Microwave cross linking study

One lot of microwave cross linked MS were produced, see Table 3.6. Lot 9709 was produced by adding 2.513 grams of 19.99% w/w BSA in ultrapure water solution to a 50 mL conical tube. To this was added 16.0 mL of 2.0% w/v CAB/DCE. The BSA solution was emulsified by vortexing on highest setting for 240 seconds.

This emulsion was microwaved (Kenmore Solid State, 900 watts, 2450 MHZ, Sears, Roebuck & Co., Chicago, IL) for approximately 15 seconds and then rotated in the oven and microwaved again for a total of 30 seconds. The microwave process had to be interrupted because of violent bubble formation (spot boiling). When finished, the tube was uncomfortably warm to the touch (approximately 45°C). After microwave exposure, the tube was vortexed for another 60 seconds (to follow the protocol used for other lots in

this series). The emulsion was agitated gently on a rotary tumbler overnight at ambient temperature.

The emulsion was made up to 50 mL by the addition of acetone and left on the tumbler for another 24 hours. This was spun down for 5 minutes at 3,000 rpm to yield clear supernatant over a tight pellet. The supernatant was decanted and the pellet resuspended in 50 mL of fresh acetone and centrifuged again (5 min. at 3,000 rpm). Again the clear supernatant was decanted and the acetone rinse repeated one more time.

After decanting, 5.0 mL of 1% w/w aqueous BSA solution was added and the pellet resuspended; this was done to cap any free aldehyde groups with BSA. The sample was returned to the rotary tumbler for 90 minutes. The tube was spun down (5 min. at 3,000 rpm) and the clear supernatant decanted. The suspension volume was made up to 50 mL by adding acetone and agitated briefly by hand. The tube was spun down (5 min. at 3,000 rpm) to yield clear supernatant over a tight pellet; the supernatant was discarded. This rinse procedure was repeated two more times using 30 mL of acetone and then the pellet was left covered with filter paper to air dry for three days. Final product weight was taken, no diameter measurements were made.

3.3 Methods: Drug Content and Elution

3.3.1 Methotrexate Studies

Methotrexate content for each MS lot was calculated by a solution loss method. The amount of MTX or Na-MTX added to a lot was known and both drugs are negligibly soluble in acetone. Thus, the amounts lost during the aqueous BSA rinse were measured and subtracted from the initial amount.

By the method of Knepp, the absorbances of a serial dilution of MTX (or Na-MTX) were measured at 305 nm versus a PBS blank in a dual beam spectrophotometer.[40] The serial dilutions covered a range of 0.00 to 0.10 mg/mL and provided a standard curve which was calculated by straight line regression. Standard curves were made for both MTX and Na-MTX and were used to calculate the drug content of the post-capping supernatant and the supernatants for the elution assay described below. Dilutions of the supernatants were made when the measured values fell significantly outside the range of the standard curve.

The elution of drug from the microspheres into PBS with time was studied at physiologic temperature, 37°C. Suspensions of drug loaded microspheres were prepared at a concentration of 10.0 mg/mL by weighing approximately 20 mg (on a four place digital balance) into 3.0 mL syringe bodies

and adding a sufficient amount of PBS to make the desired concentration. All lots were adjusted for a presumed 5% moisture content. Microspheres were suspended by passing between two syringes until a uniform suspension was achieved (usually less than five passes). The suspensions were transferred to labeled styrene test tubes and capped. The tubes were then placed in a 37°C shaker bath and incubated.

At two hours, the tubes were removed and centrifuged for 5 minutes at 3,400 rpm. The tubes were weighed before and after supernatant was scrupulously removed and retained. Fresh PBS, of equivalent mass to the supernatant removed, was added to each tube. The tubes were replaced in the shaker bath. This sampling procedure was repeated at 9, 24, 48, and 72 hours (some studies terminated at 48 hours). Retained supernatants were stored in labeled, capped styrene test tubes at room temperature until analyzed.

The supernatants had to be diluted, most often 1:100, with PBS before being read for absorbance at 305 nm versus a PBS blank in a dual beam spectrophotometer. Further dilutions were made when measured values fell significantly outside the range of the standard curves and were accounted for in calculating the drug content.

When calculating the cumulative elution of methotrexate, the three values for each supernatant were

averaged. This average value for a given time point was added to the sum of the average values for the preceding time points to get a cumulative value for the given time point.

3.3.2 Protein Elution

Protein elution measurements were performed on the supernatants taken in the previous studies. As well, an elution study was performed on the ferric ion stabilized BSA lots (9637 and 9638) and the BSA/HA lot, 9641.

The Bio-Rad protein assay was used for protein content determination. Briefly, this assay is a modification of the Bradford method.[10] It relies on the binding of a dye; the absorbance maximum of which shifts with protein binding in acidic media.

A serial dilution of the same BSA used in MS synthesis was made in PBS in the range of 0.0 to 1.0 mg/mL prior to each protein assay. Assay of this dilution was used to create a standard curve. While both straight line and second order regressions on the data produced curve fits (R^2) greater than 0.98, the second order regression fell almost directly on the observed values while the straight line did not. While the assay protocol posits a linear relationship between dye binding and protein concentration

in the range of the assay, there appears to be reagent depletion at the upper end of the scale. Results obtained in this research fell all across the scale, so protein concentrations were calculated using the second order regression.

The protein assay was performed in triplicate using the Bio-Rad protocol. Briefly, assay reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts ultrapure water. The solution was filtered to remove particulate matter and used the day of preparation (it has a two week shelf life at room temperature). For each supernatant tested, 2.0 mL of assay reagent was pipetted into each of three disposable spectrophotometer cuvettes. Into each cuvette was pipetted 40 µL of the supernatant and the contents were well mixed by repeated pipette tip flushes. These cuvettes were allowed to incubate for more than five minutes at room temperature. The cuvettes were read for absorbance at 595 nm versus a assay reagent/PBS blank in a dual beam spectrophotometer. Dilutions of supernatant were made with PBS when measured values fell significantly outside the range of the standard curve and the dilutions accounted for in calculating the protein content.

When calculating the cumulative elution of protein, the three values for each supernatant were averaged. This average value for a given time point was added to the sum of the average values for the preceding time points to get a cumulative value for the given time point.

3.3.3 Novantrone[tm] Studies

Drug content for NOV loaded microspheres was determined by a solution depletion method. An aliquot of the initial soaking solution was serially diluted and read against water at 610 nm in a dual beam spectrophotometer. The serial dilutions were 1:500, 1:1000, ca. 1:2000, and 1:4000 (the last for 9201 study only). This produced a standard curve by which the post-soak supernatant, the post-capping supernatant for lot 9308, and the acetone-rinse supernatants could be measured. Supernatants were diluted as needed to get readings that fell inside the range of the standard curve and the dilution factors were taken into account in calculating drug content. The acetone-rinse supernatants were all diluted 1:10.

The concentrations measured for each supernatant were multiplied by the volume of supernatant retrieve (accounting for dilutions) and results summed up for each batch. This was subtracted from the initial drug content of the soaking

solution for that lot and the difference was assumed to have been taken up by the microspheres. Drug content was then calculated as a percent of the mass of drug laden MS that was measured after drying. It should be noted that the NOV received from Lederle was labeled as 82.47% mitoxantrone (balance unspecified). Figures reported here for drug content take this factor into account. No *in vitro* drug or protein elution studies were performed on NOV loaded MS.

3.3.4 Acid Phosphatase Elution Studies

The presence and activity of acid phosphatase was determined by use of Sigma Diagnostic[tm] procedure #104.[61] This is a colorimetric assay in which absorbance correlates to enzyme activity. In brief, the assay involves incubating the 0.2 mL test samples in the presence of a 1.0 mL of substrate/buffer solution at 37°C for 30 minutes. The enzyme cleaves p-nitrophenol from the substrate in amounts proportional to the activity of the enzyme. The reaction is quenched and the color is developed by the addition of 0.1 M NaOH solution. The color is then stable for several hours and is read in a spectrophotometer at 410 nm. This assay is used to determine the concentration of acid phosphatase in human serum and, thus, is insensitive to the presence of serum albumin or isotonic saline.

The elution of enzyme was examined in two separate studies. The assay protocol cautions the user that this enzyme is heat labile.[61] Thus, the first study was run at 5°C As the samples were in a refrigerator, this study was done without agitation. A control solution of fresh enzyme was prepared by dissolving 1.7 mg in 34.0 mL of phosphate buffered saline (PBS), sample and enzyme concentrations are listed in Table 3.7. A 0.2 mL specimen was taken right after preparation and frozen. The remaining solution was placed in the refrigerator. A suspension of lot 9616 was prepared by suspending 85.2 mg in 21.3 mL of PBS. A 0.2 mL specimen of suspension was frozen right after preparation. As well, 1.0 mL was spun down (5 min. at 3,800 rpm) and a 0.2 mL specimen of the clear supernatant was frozen. The same was done for a suspension of lot 9617 with the exception that 42.2 mg of MS was suspended in 21.1 mL of PBS.

Table 3.7 Sample and enzyme concentrations for the first enzyme elution study performed at low temperature.

Sample	**Microsphere Mass (mg)/mL PBS**	**Enzyme Mass (μg)/mL PBS**
Acid Phos. (control)	N/A	50 μg/mL
Lot 9616	4.0 mg/mL	154 μg/mL
Lot 9617	2.0 mg/mL	149 μg/mL

At 2, 9, and 24 hours, 0.2 mL specimens of the control solution were taken and frozen. At these same time points, the suspensions were agitated briefly by hand and 1.0 mL aliquots were spun down (5 min. at 3,800 rpm). Again, 0.2 mL specimens of the clear supernatant were taken and frozen. At the 24 hour time point, the supernatants were removed completely and the pellet resuspended to their original suspension concentrations using fresh PBS. A 0.2 mL specimen of each new suspension was also frozen.

Finally, 1.0 mL aliquots of the control solution and each suspension were heated for 60 minutes in a water bath maintained at 60 to 65°C. When finished, 0.2 mL specimens of each were also frozen. When all the specimens had been collected, the assay reagents were prepared; specimens thawed; and the assay was run on the entire set.

A second elution study was performed with the following differences. First, the study was run at room temperature (24°C at the start). Second, each specimen was run in triplicate. Third, at each time point, 1.0 mL aliquots of each suspension were spun down (5 min. at 3,800 rpm) and the three 0.2 mL specimens of the supernatant were taken and frozen. The remaining supernatant was removed and each pellet resuspended in fresh PBS to its original suspension concentration and three 0.2 mL specimens of each suspension

were frozen. This was done so that the eluted and the retained activity could be assayed at each time point. Finally, a suspension of lot 9614 was run along with lots 9616 and 9617.

The control solution for the second study used 2.1 mg of acid phosphatase in 42.029 g of PBS, see Table 3.8 for suspension and enzyme concentrations. For lot 9614, 16.0 mg of MS were suspended in 32.067 g PBS. For lot 9616, 40.4 mg of MS were suspended in 10.119 g of PBS. For lot 9617, 33.1 mg of MS were suspended in 16.550 g of PBS. When all the specimens were collected, the assay reagents were prepared; the specimens thawed; and the assay run in sets of 25 specimens.

Table 3.8 Sample and enzyme concentrations for the second enzyme elution study performed at room temperature.

Sample	**Microsphere Mass (mg)/mL PBS**	**Enzyme Mass (μg)/mL PBS**
Acid Phos. (control)	N/A	50 μg/mL
Lot 9614	0.5 mg/mL	142 μg/mL
Lot 9616	4.0 mg/mL	154 μg/mL
Lot 9617	2.0 mg/mL	149 μg/mL

During the first and second elution studies, samples were taken at 23 hours and placed in a water bath at elevated temperature and then assayed along with the 24 hour

samples. This was done to determine if the microspheres would shield the enzyme from heat denaturization in a detectable way. The samples from the 5°C elution study were heated for one hour at 60°C to 65°C. When assayed, there was no detectable loss of activity, so the samples from the room temperature elution study were heated at 95°C to 100°C. This second denaturization study was done in triplicate as were the samples in the second elution study.

3.3.5 DMPL Studies

Final drug uptake was determined at Ribi by Dr. Cantrell. Briefly the soaking solution retrieved after centrifugation and the subsequent water rinses were assayed for total phosphorus and total amount recovered was subtracted from the initial content of the soaking solution. The difference between the two measurements was assumed to have been taken up by the microspheres and the value reported as mass of drug divided by initial mass of sphere times 100 (i.e. microsphere weight percent).

3.4 Methods: *In Vivo* Studies

3.4.1 Lewis Lung Carcinoma

The study on the treatment of Lewis lung carcinoma (LLC) in the murine (mouse) model with Novantrone[tm] was

performed in the laboratory of Dr. H. S. Sitren of the University of Florida under the direction of Dr. J. M. Quigg. LLC is an anaplastic, squamous carcinoma that metastasizes almost exclusively to the lung with palpable lesions on the lung in as few as 20 days.[41] LLC was obtained from the National Cancer Institute and maintained by serial transplantation in male C57BL/6 mice. Tumors were harvested from these mice and divided into fragments of approximately 1 mm^3 volume.

These fragments were implanted in B6D2F mice by subcutaneous placement in the right axilla. The mice were weighed and their tumors measured with vernier calipers three times per week. On day 10, post implantation, the mice were assigned randomly into the control group or one of five treatment groups (n of 11 or 12). By day 10, tumors were prominent enough to be measured by caliper.

Table 3.9 shows the treatments received by each group. The bound drug referred to in the table is the NOV loaded lot, 9201. The dose of 2.5 mg of bound drug represents ca. 12 mg of MS in 0.15 mL of PBS (plus the 0.5 mg of dissolved NOV). Groups 3 and 4 got a second dose of drug on day 20 that was half of the original dose. Group 3 received the repeat dose in the same location as the first while group 4 received it intra peritoneally.

Table 3.9 Six treatment groups for the LLC study using both free (dissolved) and bound (lot 9201) Novantrone[tm].

Group	Treatment
1	Control, no treatment
2	Day 10, intra tumoral (i.t.) injection of 0.15 mL of PBS containing 0.5 mg free drug and 2.5 mg bound drug
3	Day 10, i.t. injection of 0.15 mL of PBS containing 0.5 mg free drug and 2.5 mg bound drug Day 20, i.t. injection of 0.15 mL of PBS containing 0.25 mg free drug and 1.25 mg bound drug
4	Day 10, i.t. injection of 0.15 mL of PBS containing 0.5 mg free drug and 2.5 mg bound drug Day 20, intra peritoneal injection of 0.15 mL of PBS containing 0.25 mg free drug and 1.25 mg bound drug
5	Day 10, i.t. injection of 0.15 mL of PBS containing 0.5 mg free drug and 2.5 mg bound drug Day 20, surgical excision of apparent tumor mass
6	Surgical excision of apparent tumor mass on day 13

Groups were housed communally and fed *ad libitum* throughout the course of the study. Surgical procedures were performed aseptically under anesthesia and NIH accepted animal use protocols were followed. When tumor mass exceeded 25% of the individual body mass, the animal was euthanized by accepted procedure.

3.4.2 Murine Ovarian Teratocarcinoma

The study on the treatment of murine ovarian teratocarcinoma (MOT) with Novantrone[tm] was performed at Ribi ImmunoChem under the direction of Dr. J. Cantrell. MOT was obtained courtesy of Dr. J. S. Berek of the UCLA School of Medicine and maintained by serial intraperitoneal transplantation of ca. 1×10^5 cells into naive female mice. Tumors cells were harvested from these mice and washed in sterile PBS (0.15 M NaCl, 5mM PO_4, pH 7.4). Tumor cell viability was assessed by trypan blue exclusion and the cell concentration adjusted with PBS to provide an i.p. inoculum of 1×10^4 viable cells in 0.2 mL of sterile PBS.

Female C3HeB/FeJ mice of body mass greater than 18 g were injected i.p. on day 0 of the study. On day 6, the mice were randomly assigned, in sets of 10, to one of eleven treatment groups, see Table 3.10. All mice were observed daily for deaths and were weighed three times per week.

Unloaded MS were used in group 11 as another negative control to evaluate the effect, if any, of the microspheres alone. Groups 5, 6, and 7 differed from groups 8, 9, and 10 in that the first set were treated with MS that had been loaded with NOV prior to capping with BSA and the second set were treated with MS that had been loaded with NOV after the capping step. This was done to determine if capping with

BSA interfered with the release of the drug from the MS. Since NOV causes albumin to coagulate, more albumin might bind in the capping step than might otherwise bind just to free aldehyde groups. Therefore, this extra bound albumin could inhibit the release of the drug.

Table 3.10 Treatment groups for the MOT study using both free (dissolved) or bound Novantrone[tm] in PBS.

Group	Treatment
1	0.2 mL PBS, control group
2	250 µg Novantrone[tm] dissolved in 0.2 mL PBS
3	125 µg Novantrone[tm] dissolved in 0.2 mL PBS
4	63 µg Novantrone[tm] dissolved in 0.2 mL PBS
5	250 µg NOV bound in MS (lot 9308) in 0.2 mL PBS
6	125 µg NOV bound in MS (lot 9308) in 0.2 mL PBS
7	63 µg NOV bound in MS (lot 9308) in 0.2 mL PBS
8	250 µg NOV bound in MS (lot 9307) in 0.2 mL PBS
9	125 µg NOV bound in MS (lot 9307) in 0.2 mL PBS
10	63 µg NOV bound in MS (lot 9307) in 0.2 mL PBS
11	1 mg unloaded MS (lot 9306) in 0.2 mL PBS

CHAPTER 4
RESULTS AND DISCUSSION

While the chapter on materials and methods was broken down by processing steps and analytical procedures, the results and discussion will be presented by the areas of research: 4.1) methotrexate studies, 4.2) Novantrone[tm] studies, 4.3) acid phosphatase studies, 4.4) DMPL studies, and 4.5) miscellaneous MS synthesis studies. This is done for the sake of continuity.

4.1 Methotrexate Studies

4.1.1 MS Synthesis

The results for the glutaraldehyde cross linked lots of MTX loaded microspheres are presented in Table 4.1. The percent yield is calculated as the final mass of MS obtained divided by the mass of all components used to make that lot. If there was little mass lost to processing and the drying was incomplete, then it is possible to get a percent yield in excess of 100% w/w. The mean diameters are reported in microns $\pm$ sample standard deviation (n = 100).

The drug content was calculated based on initial drug mass less the drug lost to processing (see methods). The drug content is presented as the calculated residual mass of drug divided by the theoretical 100% yield mass. The protein content is calculated as the percent mass remaining when drug mass and cross linker mass are subtracted.

Table 4.1 Percent yield, mean diameter, drug content and protein content for glutaraldehyde cross linked, methotrexate and sodium methotrexate loaded MS. Mean diameter in microns ± sample standard deviation (n = 100).

Lot #	Yield %	Mean Diameter ± S.S.D.	Drug Type	Drug Content	Protein Content
9618	dissol.	N/A	N/A	0	100%
9619	110%	2.7 ± 1.9	N/A	0	98%
9620	111%	2.7 ± 2.4	N/A	0	96%
9621	89%	2.8 ± 4.0	MTX	35%	65%
9622	104%	2.6 ± 1.9	MTX	30%	70%
9623	103%	3.1 ± 2.5	MTX	47%	53%
9624	103%	3.6 ± 3.9	MTX	47%	53%
9628	19% *	2.6 ± 1.8	Na-MTX	20%	79%
9629	119%	2.5 ± 1.6	Na-MTX	26%	74%
9630	110%	3.0 ± 4.5	Na-MTX	32%	68%
9631	123%	2.6 ± 2.4	Na-MTX	37%	63%

* processing accident contributed to low yield

The yields for all lots, except 9621 and 9628, are in excess of 100%. A processing accident, in which part of the pellet was lost during decanting, contributed to the very

low yield on lot 9628. The excess yield on the other lots is attributed to incomplete drying. The MS used in the drug elution experiments and the protein elution experiments were presumed to have a 5% moisture content and the masses adjusted accordingly.

The percent yields, drug contents and protein contents for the lots cross linked with ferric nitrate nonahydrate are shown in Table 4.2. The presented values were calculated as described above.

Table 4.2 Percent yield, drug content and protein content for ferric nitrate nonahydrate cross linked, methotrexate and sodium methotrexate loaded MS.

Lot #	Yield %	Drug Type	Drug Content	Protein Content
9637	85%	N/A	0	81%
9638	92%	N/A	0	89%
9701	101%	MTX	29%	63%
9702	87%	MTX	27%	60%
9703	100%	MTX	46%	48%
9704	89%	MTX	43%	47%
9705	94%	Na-MTX	19%	67%
9706	89%	Na-MTX	26%	59%
9707	113%	Na-MTX	23%	68%
9708	98%	Na-MTX	32%	53%

Only two lots in this series had yields in excess of 100%, the more complete drying can be attributed to low humidity in the laboratory at the time of drying. It should be noted that all of the low ferric ion content lots (i.e. 9638, 9701, 9703, 9705, and 9707 had higher yields than their partner lots (same initial drug loading but higher ferric ion content). This might be due to the lower cross link density MS being able to swell more than the higher cross link density MS.

Comparing lots loaded with MTX, the use of ferric ion as the cross linking agent does not appear to have affected the final drug loading. At low loading levels, the ferric ion stabilized spheres do appear to have slightly lower loading. Comparing Na-MTX loaded MS, the ferric ion cross linked MS had much lower drug retention at the higher loading levels.

The lower retention for the Na-MTX loaded MS is not surprising given the far greater solubility (>50:1) of the sodium salt. Alteration in the manufacturing process to minimize the amount of aqueous BSA used for capping pendent aldehydes would cut down on the loss.

4.1.2 MTX Elution

The results of the methotrexate elution studies, both MTX and Na-MTX, are depicted graphically in Figure 4.1 and Figure 4.4. All of the MS suspensions for these studies

were made up to 10.0 mg/mL, thus all of the initial drug contents for these suspensions were different (ranging from 2.65 to 4.68 mg/mL for MTX loaded MS and from 1.93 to 3.72 mg/mL for Na-MTX loaded MS). To allow for ready comparison, the cumulative eluted drug for each lot was normalized to a percent of the initial amount in suspension. The percent drug content value listed in Tables 4.1 and 4.2 divided by 10 will give the initial drug content in mg/mL in the elution study. This value times the percent eluted drug value taken from the graph will give the cumulative drug eluted in mg/mL.

Figure 4.1 shows the methotrexate eluted with time in hours of exposure to PBS at 37°C for glutaraldehyde cross linked MS. The low loading MS lots, 9621 and 9622, elute 50% of their initial loading between 2 and 9 hours while the high loading MS lots, 9623 and 9624, reach half depletion between 9 and 24 hours. That both curves in each loading pair lie almost on top of one another indicates that level of glutaraldehyde cross linking has little effect on the elution of MTX from BSA microspheres. The difference in the percent elution for the low drug content pair (9621 and 9622) does become significant at the longer elution times with the high cross link lot, 9622, eluting less, see Table 4.3.

Figure 4.2 shows the methotrexate eluted with time in hours of exposure to PBS at 37°C for ferric nitrate nonahydrate cross linked MS. The low loading MS lots, 9701 and 9702, elute 50% of their initial loading between 9 and 48 hours while the high loading MS lots, 9703 and 9704,

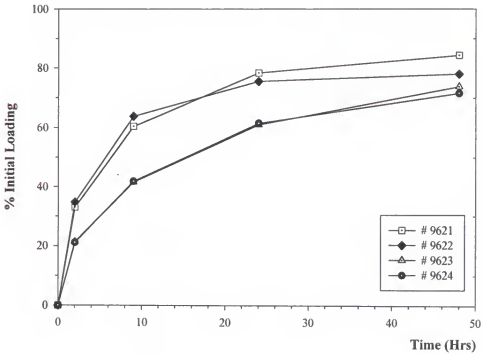

Figure 4.1 Elution of MTX from glutaraldehyde cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9621 and 9622 versus 9623 and 9624) for low and high cross link level (odd versus even).

Table 4.3 P values for comparisons of percent elution of initial loading of MTX from glutaraldehyde cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour
9621 to 9622	0.176	0.037	0.049	0.003
9623 to 9624	0.761	0.808	0.685	0.176
9621 to 9623	0.001	0.001	0.001	0.003
9622 to 9624	0.004	0.001	0.003	0.001

reach half depletion between 24 and 48 hours. Unlike glutaraldehyde cross linked MS, there is fair separation between the elution curves, although the difference between the high drug loading lots, 9703 and 9704, is not significant at later time points, see Table 4.4.

The high cross link level MS fall below the low cross link level MS. This slowing in the drug release and cross link level dependence could be caused by ferric ion binding of the drug to itself and to the stabilized matrix.

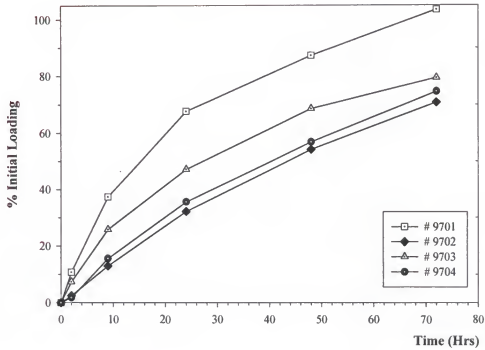

Figure 4.2 Elution of MTX from ferric nitrate cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9701 and 9702 versus 9703 and 9704) for low and high cross link level (odd versus even).

Table 4.4 P values for comparisons of percent elution of initial loading of MTX from ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour	72 hour
9701 to 9702	<0.001	<0.001	<0.001	<0.001	<0.001
9703 to 9704	<0.001	0.020	0.068	0.083	0.330
9701 to 9703	<0.001	0.015	0.024	0.037	0.024
9702 to 9704	0.101	0.020	0.028	0.041	0.013

Figure 4.3 shows the sodium methotrexate eluted with time in hours of exposure to PBS at 37°C for glutaraldehyde cross linked MS. All lots lose 50% of their loading in less than two hours. Unlike the MTX loaded MS, the low cross link level MS lots, 9628 and 9630, elute nearly 100% of their initial loading before 2 hours while the high cross link level MS lots, 9629 and 9631, level off between 70% and 80% by 24 hours. The curves in each cross link level pair are significantly different, see Table 4.5, which indicates that level of glutaraldehyde cross linking has an effect on the elution of this very soluble version of methotrexate, with a higher cross link level impairing elution (as expected).

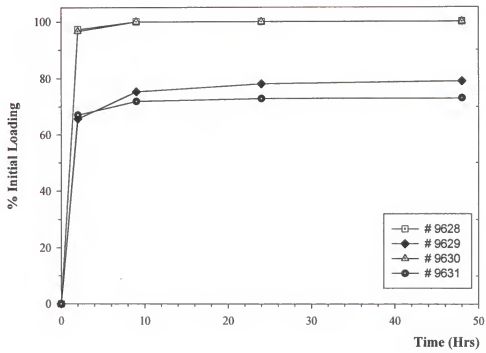

Figure 4.3 Elution of Na-MTX from glutaraldehyde cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9628 and 9629 versus 9630 and 9631) for low and high cross link level (even versus odd).

Table 4.5 P values for comparisons of percent elution of initial loading of Na-MTX from glutaraldehyde cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour
9628 to 9629	<0.001	<0.001	<0.001	<0.001
9630 to 9631	<0.001	<0.001	<0.001	<0.001
9628 to 9630	0.655	0.005	0.002	0.002
9629 to 9631	0.305	0.041	0.011	0.008

Figure 4.4 shows the sodium methotrexate eluted with time in hours of exposure to PBS at 37°C for ferric nitrate nonahydrate cross linked MS. First it should be noted that all lots appear to have eluted more drug than the initial suspensions contained (greater than 100% elution). The result cannot be attributed to experimental error and appears to be a function of the ferric ion interaction with the sodium salt of methotrexate. Having acknowledged this, the results are presented as calculated and should be used in a relative, rather than absolute sense.

As with the glutaraldehyde cross linked Na-MTX MS, the low cross link level lots, 9705 and 9707, eluted the bulk of their initial loading in less than 2 hours while the high cross link level lots, 9706 and 9708, took between 9 and 24 hours. The separation in the curves is significant (except for low cross link level at times greater than 9 hours, see Table 4.6) which indicates that both the level of ferric ion cross linking and initial loading level has an effect on the elution of Na-MTX from BSA microspheres.

Again, the slower rise in the cumulative elution of drug, relative to the glutaraldehyde cross linked, Na-MTX loaded MS, could be caused by the ferric ion binding of drug to itself and to the matrix material. MTX has two pendant carboxylic groups which would complex with the ferric ion just as would carboxylic groups on the BSA molecule.

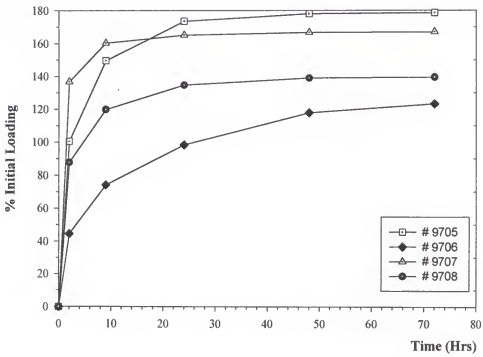

Figure 4.4 Elution of Na-MTX from ferric nitrate cross linked BSA MS versus time in hours of exposure to PBS at 37°C for low and high drug loading levels (9705 and 9706 versus 9707 and 9708) for low and high cross link level (odd versus even).

Table 4.6 P values for comparisons of percent elution of initial loading of Na-MTX from ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour	72 hour
9705 to 9706	<0.001	<0.001	0.002	0.003	0.003
9707 to 9708	<0.001	0.001	0.002	0.002	0.002
9705 to 9707	0.001	0.035	0.106	0.057	0.052
9706 to 9708	0.003	<0.001	<0.001	0.002	0.005

4.1.3 Protein Elution

The results of the protein elution studies are shown graphically in Figure 4.5 through Figure 4.8. As with the methotrexate elution results discussed above, the protein elution results are presented as the cumulative amount eluted as a percent of initial amount suspended so that lots can be compared. Figure 4.5 shows the cumulative elution of protein from MS lots with low level glutaraldehyde cross linking.

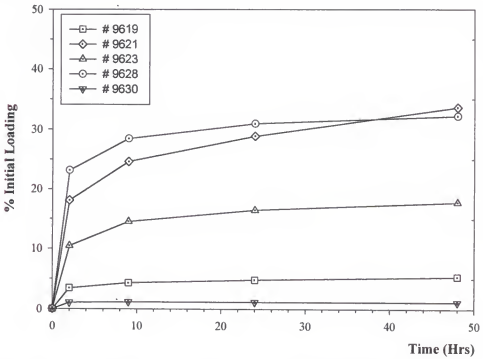

Figure 4.5 Elution of protein from BSA MS of low glutaraldehyde cross linked level versus time in hours of exposure to PBS at 37°C for unloaded (9619), MTX loaded (low 9621, high 9623), and Na-MTX loaded (low 9628, high 9630) lots.

Table 4.7 P values for comparisons of percent elution of initial loading of protein from lots with low cross link level using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour
9619 to 9621	<0.001	<0.001	<0.001	<0.001
9619 to 9623	<0.001	<0.001	<0.001	<0.001
9619 to 9628	<0.001	<0.001	<0.001	<0.001
9619 to 9630	<0.001	<0.001	<0.001	<0.001
9621 to 9623	<0.001	<0.001	<0.001	<0.001
9628 to 9630	<0.001	<0.001	<0.001	<0.001
9621 to 9628	0.003	0.008	0.023	0.050
9623 to 9630	<0.001	<0.001	<0.001	<0.001

Each of these plots is significantly different from the others with the exception of the low cross link lots, 9621 and 9628, at 48 hours, see Table 4.7.Unloaded MS, lot 9619, eluted ca. 3% of initial protein content by 2 hours and continued to rise slowly out to 48 hours. As expected, low level drug loading (both MTX and Na-MTX) caused the MS to be less stable with ca. 20% of initial protein content eluted by 2 hours and shallower rise out to 48 hours. Unexpectedly, the higher drug loadings showed more stability than the lower drug loadings. A possible mechanism might be that local high concentrations of eluting drug make the BSA less soluble (a local isotonicity effect). However, if this were

true, one would expect protein elution from lot 9630 to rise dramatically at 9 hours when much of the Na-MTX would have been eluted.

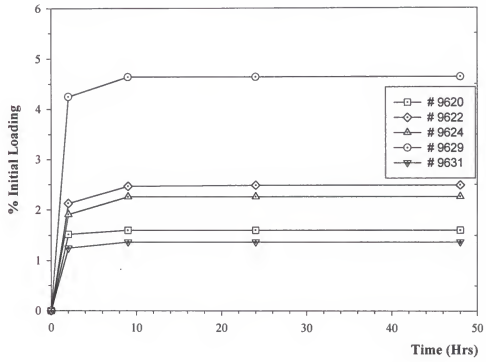

Figure 4.6 Elution of protein from BSA MS of high glutaraldehyde cross linked level versus time in hours of exposure to PBS at 37°C for unloaded (9620), MTX loaded (low 9622, high 9624), and Na-MTX loaded (low 9629, high 9631) lots.

Figure 4.6 shows the cumulative elution of protein from MS lots with high level glutaraldehyde cross linking. Each of these curves is significantly different from the others, see Table 4.8. Unloaded MS, lot 96120, eluted ca. 1.5% of initial protein content by 2 hours and essentially stopped

eluting protein. The pattern seen in Figure 4.5 (with low loading Na-MTX being least stable; either loading of MTX being less stable than unloaded; and high loading of Na-MTX being more stable than unloaded) is repeated here.

Table 4.8 P values for comparisons of percent elution of initial loading of protein from lots with high cross link level using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour
9620 to 9622	0.001	<0.001	<0.001	0.001
9620 to 9624	<0.001	<0.001	<0.001	<0.001
9620 to 9629	<0.001	<0.001	<0.001	<0.001
9620 to 9631	<0.001	<0.001	0.005	0.001
9622 to 9624	0.004	0.002	0.001	<0.001
9629 to 9631	<0.001	<0.001	<0.001	<0.001
9622 to 9629	<0.001	<0.001	<0.000	<0.001
9624 to 9631	0.001	<0.001	<0.001	<0.001

Unlike the previous graph, all of these curves flatten out at 9 hours and the least stable lot, 9629 is still more stable than 9619, low glutaraldehyde cross linking. The protein loss at low levels of glutaraldehyde cross linking is most likely indicative of actual sphere dissolution, whereas protein loss seen at high levels of glutaraldehyde cross linking may be nothing more than loss of adsorbed BSA from the capping step of MS synthesis.

Figure 4.7 shows the cumulative elution of protein from MS lots with low level ferric nitrate nonahydrate cross linking. Each of these curves is significantly different from the others, with the exception the two MTX lots, 9701 and 9703, see Table 4.9. Unloaded MS, lot 9638, eluted ca. 20% of initial protein content by 2 hours and continued to rise out to 48 hours.

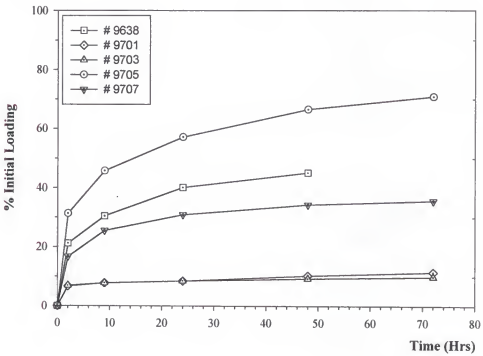

Figure 4.7 Elution of protein from BSA MS of low level ferric nitrate cross linking versus time in hours of exposure to PBS at 37°C for unloaded (9638), MTX loaded (low 9701, high 9703), and Na-MTX loaded (low 9705, high 9707) lots.

Table 4.9 P values for comparisons of percent elution of initial loading of protein from low ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour	72 hour
9638 to 9701	<0.001	<0.001	<0.001	<0.001	N/A
9638 to 9703	<0.001	<0.001	<0.001	<0.001	N/A
9638 to 9705	0.030	0.014	0.011	0.007	N/A
9638 to 9707	<0.001	<0.001	<0.001	<0.001	N/A
9701 to 9703	0.258	0.863	0.964	0.005	0.002
9705 to 9707	0.015	0.008	0.005	0.003	0.003
9701 to 9705	0.005	0.002	0.001	0.001	0.001
9703 to 9707	<0.001	<0.001	<0.001	<0.001	<0.001

Unexpectedly, the addition of drug appear to have stabilized the protein MS, with only low loading Na-MTX (lot 9705) being less stable than the unloaded MS. The MTX loaded lots (9701 and 9703) were remarkably stable with a rapid initial loss of protein and then a gradual rise out to 72 hours.

Figure 4.8 shows the cumulative elution of protein from MS lots with high level ferric nitrate nonahydrate cross linking. Each of these curves is significantly different from the other with the exception of the MTX loaded lots at 72 hours, see Table 4.10. Unloaded MS, lot 9637, eluted ca. 5% of initial protein content by 2 hours and continued to

rise slowly out to 48 hours. As expected, the addition of drug destabilized the protein MS, with all drug loaded lots being less stable than the unloaded MS lot. The MTX loaded lots (9702 and 9704), again, were more stable than Na-MTX loaded lots, with a rapid initial loss of protein and then a gradual rise out to 72 hours. The higher Na-MTX loaded lot (9708) was far less stable than the other lots.

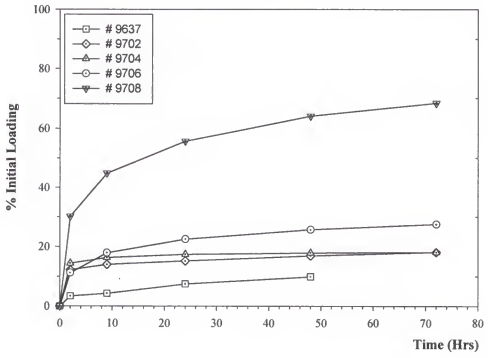

Figure 4.8 Elution of protein from BSA MS of high level ferric nitrate cross linking versus time in hours of exposure to PBS at 37°C for unloaded (9637), MTX loaded (low 9702, high 9704), and Na-MTX loaded (low 9706, high 9708) lots.

Table 4.10 P values for comparisons of percent elution of initial loading of protein from high ferric nitrate cross linked lots using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour	72 hour
9637 to 9702	<0.001	<0.001	<0.001	<0.001	N/A
9637 to 9704	<0.001	<0.001	<0.001	<0.001	N/A
9637 to 9706	<0.001	<0.001	<0.001	<0.001	N/A
9637 to 9708	0.001	<0.001	<0.001	<0.001	N/A
9702 to 9704	<0.001	0.001	<0.001	0.009	0.786
9706 to 9708	0.002	0.001	0.001	0.001	<0.001
9702 to 9706	0.003	<0.001	<0.001	<0.001	<0.001
9704 to 9708	0.003	0.001	0.001	<0.001	<0.001

4.2 Novantrone[tm] Studies

4.2.1 MS Synthesis

The results of the MS synthesis for the NOV loaded lots are shown in Table 4.11. Lot 9306 was produced as a negative control for the MOT study discussed below and, thus, had no drug content. The remaining three lots show a consistent loading of NOV in the range of 19.3% to 20.9% (mass of drug/mass of microsphere + drug). The variation in mean diameter between lots is larger than has been seen in

similar runs (see section 4.4.1, lots 9313, 9316, and 9317), but all lots produced by this particular process were in the 10 to 20 micron range.

Table 4.11 Percent yield, mean diameter, and drug content results for the MS lots produced for the Novantrone[tm] studies. Mean diameter in microns ± sample standard deviation, n = 100. Drug content in percent w/w, see text.

Lot #	Matrix Material	Capping Material	Yield %	Mean Diameter ± S.S.D.	% Drug Content
9201	BSA/PGA	none	---	---	21%
9306	BSA/PGA	BSA	77%	17.8 ± 14.9	N/A
9307	BSA/PGA	BSA	76%	10.4 ± 8.2	19%
9308	BSA/PGA	BSA	102%	13.6 ± 10.6	19%

Lots 9306 and 9307 both had percent yields in the mid 70's while lot 9308 was slightly over 100%. Most likely, this can be attributed to incomplete drying of the batch. The drug content calculations were performed on net retrieve MS mass. If this is a correct assumption, the drug content for lot 9308 (completely dry) would be on the order of 25% w/w.

4.2.2 Lewis Lung Carcinoma Study

The animal study was terminated at day 35 and all animals were euthanized. Table 4.12 shows the survival rate at day 35 by treatment group, results rounded to nearest percent point. The control group exhibited uninhibited

tumor growth. The entire group had to be sacrificed at day 21 when their tumor masses exceeded 25% of their body mass. Likewise, surgical excision alone provided short relief with 33% survival at day 35. If the study had continued, these animals would have been euthanized on day 35 anyway as their tumor masses exceeded 25% of their body mass.

Table 4.12 Percent survival at day 35 by treatment group.

Group #	Treatment	% Survival
1	Untreated control	0%
2	Inject i.t. day 10	75%
3	Inject i.t. day 10, Reinject i.t. day 20	64%
4	Inject i.t. day 10, Reinject i.p. day 20	17%
5	Inject i.t. day 10, Surgical excision day 20	92%
6	Surgical excision day 13	33%

Tumor growth was inhibited through day 28 for all animals receiving chemotherapy. The best treatment was that used on group 5 (i.t. injection day 10 followed by surgical excision on day 20). This group had 92% at day 35 with no apparent tumor mass, see Figure 4.9.

Intra tumoral treatments alone, groups 2 and 3, were less successful, yielding 75% and 64% survival respectively at day 35. What may be occurring here is the battle between delivering the chemotherapy agent to the tumor and

delivering a toxic dose systemically to the host. As discussed by Jain, blood supply to a "healthy" tumor is problematic to start with. Tissue necrosis caused by the initial injection may have rendered the second injection (in group 3) ineffective against the tumor while subjecting already weakened animals to another toxic burden. This is born out by the results in group 4. The 17% survival rate can be attributed in part to the fact that the i.p. dose had free drug in the LD_{50} concentration range for i.p. injection in mice.

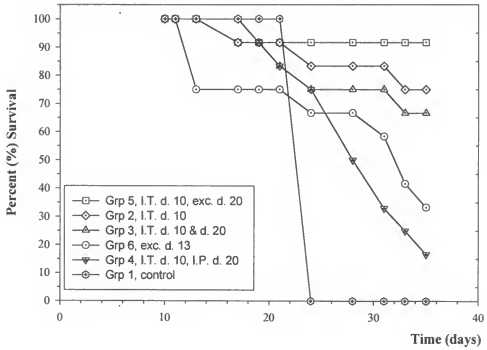

Figure 4.9 Percent survival versus time for the LLC study by treatment group.

4.2.3 Murine Ovarian Teratocarcinoma Study

The study was terminated at day 90 and all animals euthanized. Table 4.13 shows the total survivors and the percent survival at day 90. As well, it shows the mean survival time in days ± standard deviation. The P value listed is for comparison of individual treatment group mean survival times to the control group mean survival time. The P value was determined by Student's t test. The P value is the probability of the null hypothesis (no difference between a given pair of mean survival times) being true. A probability of less than 0.05 is considered to indicate a significant difference.

Figure 4.10 through Figure 4.12 show the percent survival versus time for the three treatment sets: free drug, pre-loaded microspheres (lot 9308) and post-loaded microspheres (lot 9307). In the first figure, it is seen that there is good survival out to day 90 relative to untreated controls which are all dead by day 23, see Figure 4.10. The result for the 250 μg dose is entirely unexpected given that this dosing level is well above reported LD_{50} for i.p. injections. The expected result was a much lower percent survival, perhaps as low as untreated controls.

Table 4.13 Results of the MOT study by treatment group including total survivors and percent survival at day 90; mean survival time (MST) in days ± standard deviation; and P value by Student's t test for treatment group MST compared to control group MST.

Group #	Treatment	Survive /Total	Percent Survival	MST ± S.D.	P Value
1	MOT only. No drug	0/10	0%	20 ± 4	---
2	Free drug 250 µg	10/10	100%	>> 90	≤ 0.001
3	Free drug 125 µg	4/10	40%	58 ± 14	≤ 0.001
4	Free drug 63 µg	5/10	50%	52 ± 18	≤ 0.005
5	Lot 9308 250 µg drug	9/10	90%	>> 90	≤ 0.001
6	Lot 9308 125 µg drug	4/10	40%	43 ± 10	≤ 0.005
7	Lot 9308 63 µg drug	2/10	20%	37 ± 9	≤ 0.025
8	Lot 9307 250 µg drug	8/10	80%	>> 90	≤ 0.001
9	Lot 9307 125 µg drug	8/10	80%	>> 90	≤ 0.001
10	Lot 9307 63 µg drug	1/10	10%	32 ± 8	N/S
11	Lot 9306 Unloaded MS	0/10	0%	23 ± 2	N/S

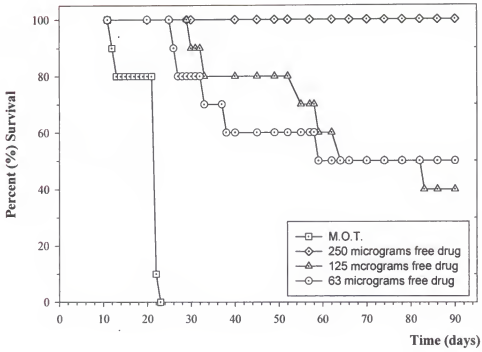

Figure 4.10 Percent survival versus time (days) post injection for untreated controls and groups 2, 3, and 4 treated with free drug (NOV) at dosages 250, 125, and 63 µg respectively.

Figure 4.11 shows the percent survival versus time, in days post injection, for: the untreated control group; the unloaded microsphere control group (lot 9306); and the groups treated with pre-loaded MS (lot 9308). Again, pre-load refers to the drug being loaded prior to the capping of pendent aldehyde groups with aqueous BSA solution. These groups were included to determine if the capping BSA layer inhibited the release of drug. In comparison to free drug, it appears that the BSA layer did inhibit the efficacy of the dose.

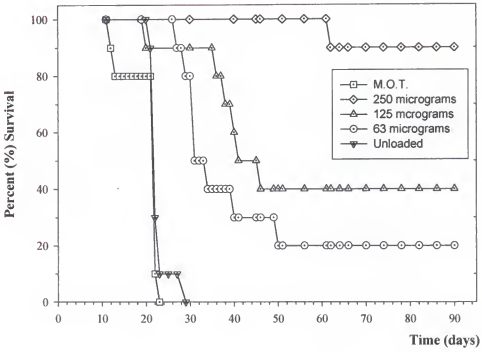

Figure 4.11 Percent survival versus time (days) post injection for untreated control, group 1; pre-loaded MS (lot 9308) groups 5, 6, and 7; and unloaded MS (lot 9306), group 11.

Figure 4.12 shows the percent survival versus time, in days post injection, for: the untreated control group; the sham control group (lot 9306); and the post-loaded MS (lot 9307). In comparison to the free drug, the drug delivered via post-loaded microspheres did quite well. Had the highest dose of free drug given the results expected, the post-load lot would have exhibited not only good survival times for the mid level dose but also shielding from systemic toxicity for the upper level dose.

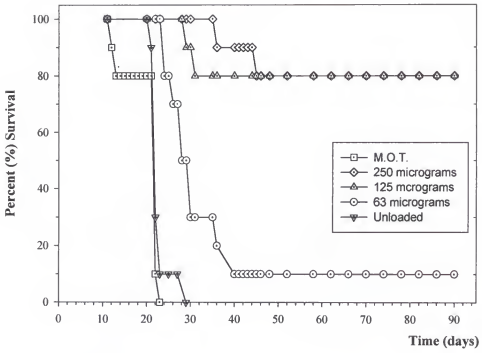

Figure 4.12 Percent survival versus time (days) post injection for untreated control, group 1; post-loaded MS (lot 9307), groups 8,9 and 10; and unloaded MS (lot 9306), group 11.

The lower level dose in both bound treatment groups was not as effective as the lower level dose of free drug. This indicates that there is some benefit to be had from quick initial release of the drug. This may explain why the pre-loaded MS did not perform as well as the post-loaded MS.

4.3 Acid Phosphatase Studies

Determination of enzyme activity was made readily by use of an assay obtained from Sigma. Once microspheres had

been produced, relatively simple yet accurate measurements were obtained by this assay method.

4.3.1 MS Synthesis

The results of the MS synthesis are shown in Table 4.14. No diameter measurements were performed on these lots as the production method was similar to many previous lots which had produced microspheres with mean diameters in the range of 3 to 5 microns (see results in section 4.1.1).

Table 4.14 Lot descriptions and yield results for lots 9613, 9614, 9616, and 9617. All masses in grams.

Lot #	Matrix Material	Loading Process	Enzyme Content	Lot Yield %
9613	BSA	N/A	N/A	89%
9614	BSA	Post Load	0.087	73%
9616	BSA	Post Load	0.024	100%
9617	BSA/Acid Phosphatase	*In Situ*	0.045	100%

4.3.2 Acid Phosphatase Elution

For ease of reference, Table 4.15 recapitulates the sampling protocol for the elution studies run on the acid phosphatase microspheres. Again, lots 9614 and 9616 were loaded after microsphere synthesis and lot 9617 was bulk loaded during microsphere synthesis.

Table 4.15 Acid phosphatase elution study protocol. Lots 9614 and 9616 were post-loaded and lot 9617 was bulk loaded.

Time	5°C, lots 9616 & 9617	24°C, lots 9614, 9616, 9617
0 hrs	Control and specimen supernatants	Control, specimen supernatants and rinsed suspensions
2 hrs	Control and specimen supernatants	Control, specimen supernatants and rinsed suspensions
9 hrs	Control and specimen supernatants	Control, specimen supernatants and rinsed suspensions
24 hrs	Control, specimen supernatants and rinsed suspensions	Control, specimen supernatants and rinsed suspensions

The activity of acid phosphatase is dependent on the time and temperature history of the enzyme as it is reconstituted from lyophilized powder. Figure 4.13 shows the enzyme activity versus time for the control solution of acid phosphatase and for the supernatants taken from suspensions of lots 9616 and 9617 in both the 5°C and 24°C elution studies.

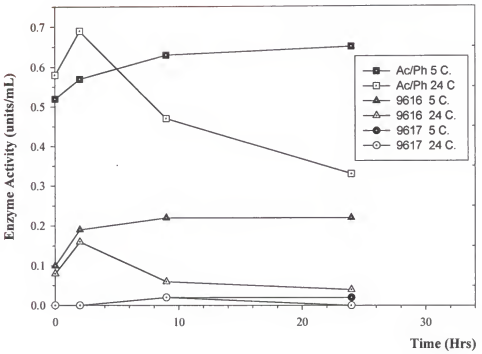

Figure 4.13 Assayed enzyme activity versus time for control solutions (50 µg/mL of acid phos./PBS) and supernatants from suspensions of lots 9616 and 9617 (approximate suspension concentrations of 150 µg/mL of acid phos./PBS) for elution studies done at 5°C and 24°C.

In each case, control and suspensions, the activity increases initially. In the low temperature study, the activity increases and then levels off. In the room temperature study, the activity climbs to a maximum value (comparable to the maximum achieved at low temperature) and then decreases. Figure 4.14 shows the eluted activity for the three lots in the room temperature study.

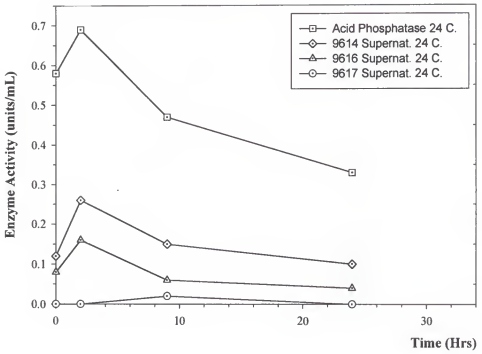

Figure 4.14 Assayed enzyme activity versus time for control solutions (50 μg/mL acid phos./PBS) and supernatants from suspensions of lots 9614, 9616, and 9617 (approximate suspension concentrations of 150 μg/mL acid phos./PBS) for elution studies done at 24°C.

Here it can be seen that lot 9614 eluted enzyme more quickly than lot 9616; this may be attributable to the nearly 10 fold higher loading of enzyme on lot 9614. Both lot 9614 and 9616 were loaded after microsphere synthesis, but lot 9614 was made 4 months prior to 9616. This indicates that loading the enzyme into BSA microspheres did not adversely affect the activity over the long term, an important and desirable result.

Figures 4.13 and 4.14 also show that very little enzyme eluted from lot 9617, which was loaded at the time of MS synthesis. Figure 4.15 shows the enzyme activity of rinsed suspensions in the room temperature study. Here it is seen that, as expected, the activity of lots 9614 and 9616 falls off with time. This can be attributed both to the decay of activity with time (as exhibited by the control solution) and to the activity lost to elution of enzyme (as seen in Figure 4.14).

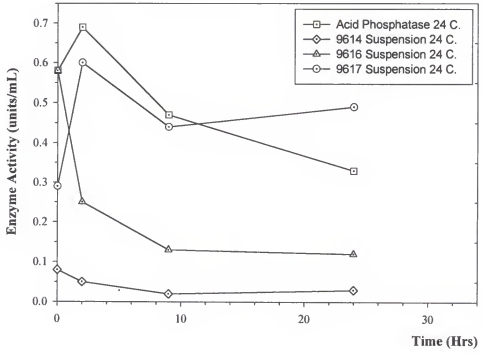

Figure 4.15 Assayed enzyme activity versus time for control solutions (50 µg/mL acid phos./PBS) and rinsed suspensions of lots 9614, 9616, and 9617 (approximate suspension concentrations of 150 µg/mL acid phos./PBS) for elution studies done at 24°C.

The activity of enzyme trapped inside the microspheres of lot 9617 remains relatively stable after its initial rise (from zero to two hours). With time at room temperature, the acid phosphatase by itself loses 43% of its initial activity and 52% of its peak activity compared to the activity at 24 hours. Whereas rinsed suspension of lot 9617 actually gains 69% from its initial activity and loses only 18% from its peak activity compared to its activity at 24 hours.

From this result, it is concluded that merely dissolving the enzyme does not render it active. The time lag between initial and peak activity indicates that there is some other reconstitution process involved. The slow rise in activity in the low temperature study indicates that this reconstitution process is temperature sensitive. It is unlikely that stoichiometry of the enzyme is changing due to the addition of PBS. Rather, it is likely that the conformation of enzyme molecules is changing from an inactive to an active one. This has bearing on the activity of the enzyme still in microspheres, in so far as some of the loss of activity might be attributable to the inability of the enzyme to change confirmation inside a matrix of cross linked albumin.

The results of the heat denaturization study are presented in Table 4.16. As can be seen here, the incorporation of enzyme into BSA microspheres did nothing to protect the activity of the enzyme at 95°C to 100°C. That activity is seen after one treatment at 60°C to 65°C is not surprising. No loss of activity was seen after 24 hours in the low temperature elution study, so it is to be expected the same would apply at 23 hours.

Table 4.16 Enzyme activity after heat treatment. Enzyme activity in units/mL (± sample standard deviation, n = 3) after 0 and 24 hours rehydration at room temperature for control solutions and rinsed suspension as well as after 23 hours at 5°C and 1 hour at 60°C to 65°C and after 23 hours at room temperature and 1 hour at 95°C to 100°C.

Sample	0 hr, 24°C	24 hr, 24°C	23 hr @ 5°C 1 hr ~60°C	23 hr, 24°C 1 hr ~95°C
Control Solution	0.58 ± 0.02	0.33 ± 0.01	0.60 (n = 1)	0.0 ± 0.0
9614 Post-load	0.08 ± 0.00	0.03 ± 0.02	N/A	0.0 ± 0.0
9616 Post-load	0.58 ± 0.00	0.12 ± 0.01	0.59 (n = 1)	0.0 ± 0.0
9617 Bulk-load	0.29 ± 0.06	0.49 ± 0.09	0.82 (n = 1)	0.0 ± 0.0

Andrews found bovine milk derived acid phosphatase to be relatively stable (greater than 60% of initial activity) for periods up to one minute as high as 78.6°C; whereas there was 90% loss of activity in 30 seconds at 90°C.[3] The high value for the bulk loaded lot, 9617, at approximately

60°C could be due a chance high reading or to temperature effect on the swelling of the cross linked BSA sphere matrix. Increased swelling would lead to greater access of the assay substrate to the trapped enzyme.

Table 4.17 Possible causes for loss of enzymatic activity for enzyme loaded onto or into BSA microspheres.

Point in Process	**Possible Mechanisms of Activity Loss**
1) During MS synthesis	a) Normal decay with time while rehydrated. b) Adverse effect of glutaraldehyde. c) Adverse effect of acetone precipitation.
2) During post-loading	a) Normal decay with time while rehydrated. b) Adverse effect of acetone precipitation.
3) During use of MS	a) Enzyme reconfirmation upon rehydration constrained by BSA matrix. b) Enzyme conjugation with BSA is sufficiently strong to shield activity.

In summary, it has been confirmed that enzyme was successfully loaded into and onto BSA microspheres. While there is definite loss of activity relative to free enzyme, assays demonstrated that active enzyme could be eluted from post-loaded MS over time and that enzyme remained active while inside or attached the spheres. The loss of enzyme

activity can be attributed to a number of factors (see Table 4.17), though it is most likely due to the normal decay in activity over time while rehydrated at room temperature. Finally, enzyme that was post-loaded and assayed four months later demonstrated activity similar to freshly post-loaded MS.

4.4 DMPL Studies

4.4.1 MS Synthesis

The MS lots produced for the DMPL studies provided a selection of materials for testing out the loading of DMPL. The results from the MS synthesis are presented in Table 4.18. Note that all lots, except 9318, made use of glutaraldehyde as the cross linking agent. Lot 9318 used ferric nitrate nonahydrate.

4.4.2 DMPL Loading

The DMPL loading was performed at Ribi by Dr. Cantrell, who also performed the content assays. The results of the loading studies are presented in Table 4.19. These results are from Dr. Cantrell's ongoing research and, for the sake of completeness, are presented here with permission.

Table 4.18 Lot composition, yield and mean diameters (μm ± sample standard deviation, n = 100) for the lots prepared for DMPL loading studies at Ribi ImmunoChem, Inc.

Lot #	Matrix Material	Capping Material	Yield %	Mean Dia. μm ± S.S.D.
9313	BSA	a-BSA	104%	11.1 ± 5.4
9316	BSA/PGA	BSA	87%	12.1 ± 7.0
9317	BSA/PGA	a-BSA	147%	12.4 ± 6.9
9318*	BSA	BSA	84%	5.4 ± 8.5
9319	BSA/PGA	BSA	98%	8.0 ± 5.8
9320	a-BSA/PGA	a-BSA	86%	6.8 ± 8.1
9404	BSA/PGA	BSA	85%	4.4 ± 7.4
9405	HAS/PGA	HSA	82%	4.1 ± 6.3

*Ferric nitrate nonahydrate cross linker

Table 4.19 Uptake of DMPL from ethanol and/or triethylamine (TEA) solution by MS as a percent of initial MS mass.

Lot #	Matrix Material	Capping Material	Percent Uptake from Ethanol	Percent Uptake from TEA
9313	BSA	a-BSA	---	44.0%
9316	BSA/PGA	BSA	70.0% & 72.4%	---
9317	BSA/PGA	a-BSA	---	41.3%
9318*	BSA	BSA	69.9%	94.0%
9319	BSA/PGA	BSA	79.6%	29.3%
9320	a-BSA/PGA	a-BSA	75.8%	54.7%
9404	BSA/PGA	BSA	74.4%	---
9405	HSA/PGA	HSA	60.8%	---

* Ferric nitrate nonahydrate cross linker

In general, the uptake of DMPL from ethanol solution appears to be greater than from triethylamine solution. The use of lipid free BSA as either a matrix material or as a capping solution does not appear to affect the uptake of DMPL from either solution. While HSA as a matrix material and capping solution does the worst in taking up DMPL from ethanol solution, it cannot be determined if this is statistically significant. As well, it is not known what effect, if any, the use of ferric nitrate as a cross linking agent had on the uncharacteristically high uptake of DMPL from triethylamine that was seen with lot 9318.

4.5 Miscellaneous MS Synthesis Studies

The results of the miscellaneous MS synthesis studies are presented below. The initial HA studies and the a-PEG studies were unsuccessful. The CMC studies, the BSA/HA trial and the microwave exposure studies all produced stable microspheres.

4.5.1 MS Synthesis

The percent yield results for the HA studies, the CMC studies, the a-PEG studies, and the microwave exposure study are presented Table 4.20. Two of the lots have percent yields in excess of 100%. As mentioned above, the most

likely explanation for this is incomplete drying. The entire preparation is performed in a single tube, so foreign body contamination is unlikely. While it is possible that there is residual CAB on the microspheres, this is also unlikely given the extensive washing in acetone (a solvent for CAB).

The yields for the HA studies are listed as 0% because of the damage done to the spheres; which is seen, under the microscope, when acetone is added to dry the spheres. The a-PEG treated microspheres dissolved during the capping step. This indicates that the expected cross linking action never took place.

Table 4.20 Percent yield results for the miscellaneous MS synthesis studies by lot designation and composition.

Lot #	Matrix Material	Cross Linker	Capping Material	Yield %
HA-1	HA	$Fe_2(SO_4)_3$	N/A	0%
HA-2	HA	$Fe_2(SO_4)_3$	N/A	0%
HA-3	HA	$Fe_2(SO_4)_3$	N/A	0%
9641	BSA/HA	glutarald.	BSA	94%
9639	CMC	$Fe(NO_3)_3$	BSA	79%
9640	CMC	$Fe(NO_3)_3$	BSA	117%
9635	BSA	a-PEG	BSA	0%
9636	BSA	a-PEG	BSA	0%
9709	BSA	microwave	BSA	106%

The CMC studies produced stable microspheres. However, there was no assay developed for testing the stability of these MS at physiologic conditions. Lack of time and funding prevented loading this system with MTX or Na-MTX, so no further testing was performed.

The microwave treated BSA emulsion produced stable microspheres. These and the BSA/HA microspheres were tested for protein elution. The results are covered in the next section.

4.5.2 Protein Elution

Protein elution studies were performed on the BSA/HA microspheres and on the microwave stabilized microspheres. The protein elution results do not reveal much about the elution of HA. However, since the HA in the starting solution is of ca. 2 MDa molecular weight, it is unlikely much HA eluted.

It should be emphasized again that is not known whether the exposure to microwave energy causes any chemical reactions in the emulsion. It is presumed that all of the microsphere stabilization imparted by microwave exposure results simply from thermal gelation caused by the heating of the emulsion.

Figure 4.16 shows the protein eluted into PBS at 37°C versus time as a percent of the initial protein content in the microsphere suspensions. Each of these curves is significantly different from the others, see Table 4.21. As is seen here, BSA/HA microspheres elute more protein than plain BSA microspheres of similar cross link (glutaraldehyde) content. This can be attributed to the presence of HA in the matrix. The swollen HA molecules would get in the way of BSA during the cross linking reaction, fragmenting the gel. As well, the presence of HA on the surface of the sphere would provide sites for simple absorption of BSA during the capping phase of processing. This BSA would decouple more readily than BSA bound to pendent aldehyde groups.

As seen in Figure 4.16, the protein elution from microwave stabilized microspheres falls between that of ferric ion cross linked and glutaraldehyde cross linked microspheres. However, the asymptotic behavior of the microwave stabilized microspheres is more similar to the glutaraldehyde treated microspheres. While ferric treated BSA appears to undergo increasing dissolution, the microwave

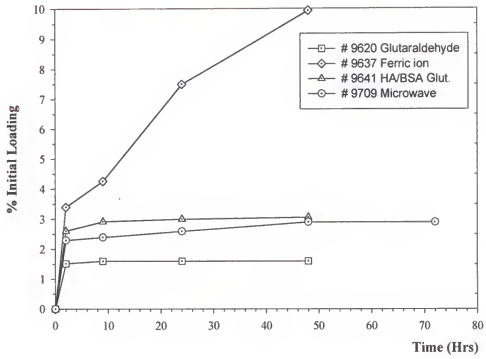

Figure 4.16 Protein elution from microsphere suspension into PBS at 37°C versus time in hours. Lots 9620 and 9641 cross linked with glutaraldehyde; lot 9637 with ferric nitrate; and lot 9709 by microwave irradiation. Normalized to a percentage of initial protein loading in suspension.

Table 4.21 P values for comparisons of percent elution of initial loading of protein from lots cross linked with glutaraldehyde, 9620 and 9641; ferric nitrate, 9637; and microwave, 9709 using the alternate Welch t Test (assumes Gaussian populations with unequal S.D.s). Null hypothesis is that the mean values of compared lots are the same at the given time point, with $P \leq 0.05$ indicating a significant difference.

Lot to Lot	2 hour	9 hour	24 hour	48 hour
9620 to 9637	<0.001	<0.001	<0.001	<0.001
9620 to 9641	0.002	0.001	<0.001	0.002
9620 to 9709	0.004	0.003	<0.001	<0.001
9641 to 9709	0.011	0.005	0.009	0.073

treated BSA remains very stable. This is to be expected since ionic complexes are seldom as stable as covalent bonds and the microwave induced, thermal gelation of BSA is, most likely, a covalent process.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Research is not unlike the Hydra of ancient myth; once one question is answered, several more spring up to take its place. There are myriad possible directions that the research discussed above could take from here and the results found on many of these paths could have value to both medicine and industry. The following conclusions and recommendations relate to areas the author feels will yield the most immediate and worthwhile results.

5.1 Methotrexate Studies

5.1.1 Conclusions

The elution studies demonstrate that both MTX and Na-MTX can be loaded into MS and retrieved in suspension. Drug loadings as high 46% w/w were achieved and there is not indication that significantly higher level could not be attained with the BSA matrix and the cross linking agents used here.

Both glutaraldehyde and ferric ion produced stable microspheres, with high level glutaraldehyde treated MS being the most stable and low level ferric ion being the

least. There appears to be strong interaction between the ferric ion and the sodium salt of methotrexate. It is not known if this interaction will have an adverse effect (if any) on the efficacy of that drug.

5.1.2 Recommendations

The original goal of this research was to find a way to deliver methotrexate in therapeutic levels (or greater) to the synovial joint, with the aim of replacing the current systemic treatment of up to 15 mg per week administered over a 24 hour period. The high loading MTX MS with ferric cross linking are the best choice for meeting these requirements. The lots created for this research eluted drug out to 72 hours and had the least protein stability. A MS delivery system designed around MTX, BSA matrix, and ferric ion cross linking would provide uniform drug delivery over several days. The residual spheres would dissolve over a slightly longer period and the breakdown products would be very acceptable in the joint.

Much more work is needed before methotrexate laden microspheres can be tested in a human study. Some *in vitro* studies must first be performed to rule out non-drug related toxicity and pyrogenicity. Once that is done, this delivery system should be tested in current animal models for

rheumatoid arthritis. Assuming no adverse reactions in animals, the studies can proceed to humans.

The human knee is a common site of complaint amongst RA sufferers and is an ideal joint for study of efficacy. The knee possesses one of the largest and the most accessible synovial capsule in the body. The adult knee contains approximately three mL of synovial fluid and can be easily cannulized, aspirated, and re-injected.

Something that must not be overlooked in the preparation for animal and human studies is the possible use of "leucovorin rescue". The systemic toxicity of MTX can be reduced by increasing the levels of reduced folate in circulation.[15] One common reduced folate used for this purpose is 5-formyl-tetrahydrofolate, or leucovorin. Leucovorin rescue is used in high dose MTX chemotherapy. Lethal doses of MTX are administered and the systemic toxicity of these doses is counteracted, after a brief interlude, by the administration of leucovorin. The reduced folate competes for the same binding sites as MTX giving the body greater time to clear the toxic agent. Proliferative cells, including the targeted cancer, are not as well shielded by this as are non-proliferative cells.

A similar protocol is proposed here. MTX laden microspheres can be injected into the afflicted synovial capsules, in what would be relatively high doses for

treatment of RA, and leucovorin can be administered systemically to provide protection to the non-target tissues. Leucovorin diffusing into the synovial capsule and circulating synovial vasculature would reduce the effectiveness of the MTX being delivered, but it would serve to localize further the action of the drug.

5.2 Novantrone[tm] Studies

5.2.1 Conclusions

The results of the LLC model indicate that MS based mitoxantrone chemotherapy avoids systemic toxicity. The amounts of bound drug given were at or in excess of the LD_{50} for this animal model. The best treatment was a combination of chemotherapy and surgical excision of the bulk of the tumor mass. However when compared to no treatment or simple surgical excision, excellent survival rates were seen in treatment with drug loaded microspheres alone.

The results of the MOT model indicate that there is reduced toxicity for mitoxantrone chemotherapy based on bound drug. The fourfold increase in mean survival time for several of the treatment groups indicates that there might have been successful cures. An initial fast release of this drug appears to be important to its efficacy and, thus, the post-loaded MS performed better than the pre-loaded.

5.2.2 Recommendations

Both of the models used to test NOV loaded MS have some implications for general cancer treatment with NOV, especially in cases where the tumor is localized but surgical excision of the tumor mass is difficult or contraindicated. Novantrone[tm] has recently received approval for a novel indication, the relief of generalized pain associated with bone cancer. While it does not improve survival, it does reduce general discomfort. Tannock *et al.* have done a study in a similar area, looking at pain reduction in hormone-resistant prostate cancer.[65] They, too, found no increase in survival but a significant decrease in pain when mitoxantrone was added to the treatment protocol. They also found several possible cases (5/130) of cardiac toxicity which could be attributed to the use of systemic mitoxantrone.

Prostate cancer is a localized cancer for which surgery is very difficult (due to the high rate of post-treatment impotence). Injection of NOV loaded MS into the prostate would allow for very high treatment concentrations locally without increasing the risk of cardiac toxicity.

5.3 Acid Phosphatase Studies

5.3.1 Conclusions

It has been confirmed that the enzyme, acid phosphatase was successfully loaded into and onto BSA microspheres. While there was a definite loss of activity after loading (relative to free enzyme), assays for activity demonstrated that active enzyme could be eluted from the surface of post-loaded microspheres over time. As well, the enzyme remained active while inside or attached to the spheres. It is most likely that the loss of activity can be attributed largely to natural loss of activity with time while rehydrated at room temperature. Finally, incorporation of the enzyme into BSA microsphere did not appear to affect the long term stability of the enzyme, with four month old, post-loaded microspheres demonstrating activity similar to freshly prepared post-loaded microspheres.

5.3.2 Recommendations

Enzyme loading should be investigated further with enzymes selected for possible therapeutic applications. Further investigation into acid phosphatase should start with performing the synthesis of bulk or in situ loaded microspheres at lower temperatures (e.g. 5°C) to verify whether or not the activity loss is due to natural degradation of the enzyme at higher temperatures (e.g. room

temperature) during microsphere synthesis. This will be useful information for preparation of other enzyme/microcarrier systems as many enzymes are either heat labile or degrade over time. Finally, the elution and activity experiments should be repeated at physiologic temperature and conditions.

5.4 DMPL Studies

5.4.1 Conclusions

DMPL loaded easily onto BSA, BSA/PGA, a-BSA/PGA, and HSA/PGA microspheres. The loading solvent (ethanol versus triethylamine) appears to be of greater import than either the matrix composition or the cross linking agent used to make the microsphere; with ethanol yielding higher DMPL uptake. The one exception to this is the high loading density achieved when loading from triethylamine onto BSA microspheres that had been cross linked with ferric ion.

5.4.2 Recommendations

Pending the results of animal studies performed with the loaded microspheres, the only work that can be suggested is to verify the effect ferric ion has on loading DMPL from triethylamine solution. Lots duplicating 9318 could be produced and run through the same loading process. As well,

lots using aluminum or chromium in place of ferric ion could also be produced.

5.5 Miscellaneous MS Synthesis Studies

Several conclusions drawn from this research reflect on topics that were investigated directly. One such instance being the problem of post-synthesis hydrophobicity of the microspheres.

5.5.1 Hydrophobicity

5.5.1.1 Observations

One very common drawback of protein microspheres is the hydrophobicity of the finished, dry product. While alluded to in the literature, hydrophobicity remains largely unexplored. Protein microspheres will eventually rehydrate when placed in contact with aqueous media however, rapid resuspension requires the addition of wetting agents or vigorous agitation. Wetting agents can pose problems with drug/device approval and the requirement for vigorous agitation necessitates additional training of the end user to insure uniform resuspension of the product.

In the case of BSA microspheres stabilized (cross linked) with glutaraldehyde, this hydrophobicity is not surprising. First, one of the functions of serum albumin is to serve as a carrier for fatty acids in the blood.[62] It would be natural to assume that the lipophilic regions on

the albumin molecule would align themselves toward the water/DCE interface of the microsphere surface. Once cross linking is achieved, the molecules are more or less constrained to this orientation, yielding a more hydrophobic end product.

Second, as mentioned in the background, the hydrated albumin molecule has positively charged groups on the surface. This would, most likely, be the result of the amine terminated amino acids being oriented towards the outside of the molecule in its normal hydrated state. Once these amines are reacted with glutaraldehyde during the stabilization (cross linking) step, the relatively hydrophilic ethyl-amine side group is replaced with what should be a relatively hydrophobic ethyl-imino-propyl linkage. This would increase the hydrophobicity of the finished microsphere.

5.5.1.2 Recommendations

The swelling of hydrated serum albumin is pH sensitive and increases with decreasing pH. Swelling these molecules before stabilizing the microsphere by glutaraldehyde cross linking should provide a more open network for rehydration of the finished product. The microsphere synthesis described here should be repeated using BSA solutions at pHs ranging from neutral down to pH 3. Lowering the pH should adversely affect the equilibrium binding of aldehyde to

lysine, but it may be possible to affect this by altering the temperature of the reaction at each pH studied.

Likewise, other dialdehydes should be investigated. Although the author was unable to achieve stabilization using aldehyde terminated PEG, this avenue of research should not be abandoned.

5.5.2 HA and CMC as Carrier Materials

5.5.2.1 Conclusions

The primary drawback of using the polysaccharides as carrier materials is that their relatively high viscosity at relatively low concentrations interferes with making uniform emulsions. When the starting solution is only a few percent carrier material, there will be very little carrier material present to act as a scaffold for binding and/or containing the drug. Considerable shrinkage during intermediate and final drying (dewatering) steps could lead to ruptured or damaged spheres as well.

This is not to say these materials should be abandoned. A mixtures of BSA and HA formed acceptable microspheres. Likewise, low molecular weight CMC formed acceptable microspheres at both high and low ferric nitrate exposure.

5.5.2.2 Recommendations

HA microspheres should continue to be investigated with emphasis on using lower molecular weight samples of this polysaccharide. The lower the molecular weight the

lower the viscosity at a given concentration. Both HA and CMC with molecular weights in the range of 50 to 100 kDa should be examined.

For CMC, one other characteristic should be investigated. Increasing the degree of substitution increases the solubility and decreases the resultant viscosity at a given concentration.[6,7] The manufacture of microspheres using CMC of a given molecular weight range should investigated while varying the degree of substitution.

Mixtures of these polysaccharides and BSA should be investigated with both cross linking systems (ferric ion and glutaraldehyde. In the glutaraldehyde system, the addition of polysaccharides could serve as a wetting agent which would decrease the problem of post-synthesis hydrophobicity.

5.5.3 a-PEG Cross Linking

5.5.3.1 Conclusions

Propionaldehyde terminated PEG did not provide any cross linking stability to the BSA microspheres at either level tested. This may have been a function of the reaction conditions or simply insufficient time to run to completion. Zalipsky comments on the low binding efficiency of aldehyde terminated PEG's in general.[71]

5.5.3.2 Recommendations

Other forms of linear PEG terminated with reactive groups could be investigated. There are a wide variety of amine reactive termination groups available (e.g. N-hydroxysuccinimide, isocyanate, and epoxide) that could be used for cross linking BSA as a carrier matrix material.

5.5.4 Microwave Cross Linking

5.5.4.1 Conclusions

Microspheres were successfully stabilized by thermal gelation induced by microwave exposure. These microspheres eluted protein at a rate comparable to both high level glutaraldehyde cross linking and high level ferric ion.

5.5.4.2 Recommendations

Microwave exposure should be investigated for use with drug that are stable up to the boiling point of the continuous phase of the emulsion (as solvent evaporation will limit the temperature rise). This method would not be useful for most enzyme loading given the thermal instability of most enzymes in aqueous solution.

The effect of mean droplet size in the emulsion on the efficiency of emulsion heating at a given microwave frequency should be examined as well. Emulsion droplets of one diameter may couple better with the microwave signal than droplets of a larger or smaller would couple. A crude first experiment would be to look at temperature rise times

for a given mass of emulsion (both continuous and dispersed phases held constant) at a given frequency with different mean droplet sizes.

APPENDIX
CHARACTERIZATION OF BIOPOLYMER FLUIDS

Viscosity is a characteristic of materials which can be used in both the prospective and retrospective analysis of implant performance. This appendix covers the prospective analysis of a new material for prevention of post-operative adhesions and the retrospective analysis of breast implant gels both from unused and from explanted implants.

A.1 Fluorinated Polyethyleneglycol

A.1.1 Introduction

Adhesion formation in the abdominal cavity subsequent to trauma, disease or foreign body reaction brings about severe discomfort and even life threatening bowel obstruction.[25] It has been well demonstrated that the introduction of viscous polymer solutions into the abdominal cavity prior to surgical manipulation significantly reduces both the incidence and severity of post-operative adhesion formation.[13, 26, 69] It is believed that the viscous polymer solutions shield the abdominal tissues is several ways. First, these solutions prevent desiccation while the tissues

are exposed during the surgical procedure. Second, the lubricious nature of these solutions mitigates the trauma to delicate tissues imparted by the handling of tissues during the procedure. Finally, depending on the polymer used to make the solution, the solutions may serve as a physical barrier between damaged tissue surfaces during the crucial early stages of clot formation and tissue healing.

A variety of water soluble polymers have been tested for their adhesion prevention properties; including hyaluronic acid (HA), carboxymethylcellulose (CMC), polyvinylpyrrolidone (PVP), and polyethyleneglycol (PEG). The efficacy of the solutions appears to be correlated to the solution concentration. In a study using HA solutions in a rat cecal abrasion model, both the adhesion incidence decreased and the incidence of no adhesion formation increased as the concentration of HA is solution increased.[13] This affect is seen with other polymers, e.g. CMC, and it may be solution viscosity which provides the shielding effect (with more viscous solutions providing a more tenacious protective coating).

There is a problem with using high molecular weight PEG's in so far as the residual monomer contamination of ethyleneglycol is toxic and can cause death.[12] While low molecular weight PEG can be purified to reduce this problem, the concentration of low molecular weight PEG needed to

achieve the desired viscosity is prohibitive (in excess of 20% w/v). It is desirable to minimize both cost and possible insult to the patient.

Termination of both ends of a low molecular weight PEG molecule with perfluoroalkane moieties creates an interesting water soluble polymer (f-PEG), see Figure A.1.[49] The perfluoroalkane end groups are extremely hydrophobic and tend to self aggregate. This microscopic phase separation creates shear labile crosslinks in the solution. Thus, high viscosity solutions of low molecular weight f-PEG can be made at relatively low concentrations.

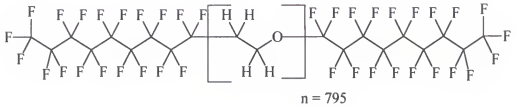

Figure A.1 Structure of perfluorooctane terminated poly-ethyleneglycol.

A.1.2 Materials and Methods

MATERIALS: The perfluoroalkane terminated PEG used for these studies was comprised of a 35 kDa linear PEG molecule terminated with either C_6F_{13} or C_8F_{17} end groups. This material was supplied courtesy of Dr. Steve Menchen of Perkin-Elmer, moisture content 2.9% w/w and was used without further purification or modification. High molecular weight (~5 MDa) PEG was obtained from Sigma. For all other materials, see section 3.1.3 of this dissertation.

SOLUTION PREPARATION: High molecular weight PEG solutions were prepared at various concentrations. Dry polymer (accounting for moisture content) was added to phosphate buffered saline (PBS) and dissolved in a covered beaker by stirring overnight with a magnetic stir bar/stir plate. The resulting solution was filtered through a 3.0 μm non-woven mesh into glass crimp top vials and autoclaved ("20 minute" slow exhaust cycle, 121°C). The samples were sealed and stored in the refrigerator prior to use or testing.

Due to the small quantity of f-PEG available, solutions were prepared by adding the desired amount of dry polymer (accounting for moisture content) to the appropriate amount of PBS and passing between two 60 mL syringes until a

uniform solution was obtained. The resulting solutions were filtered through a 3.0 μm non-woven mesh into glass crimp top vials. These solutions were prepared both with and without autoclaving as the polymer degraded with heating. Samples used in the animal studies were prepared aseptically at 1.1% w/v (of the C_8F_{17} terminated f-PEG) using sterile equipment and materials (other than the f-PEG) and used without autoclaving.

SOLUTION ANALYSIS: Viscometry on these solutions was performed on a cone/plate viscometer (RVTDV-IICP, Brookfield Engineering Laboratory, Inc., Stoughton, MA) using #40, #51, and #52 cones. The plate was water jacketed and maintained, via a water bath, at 25°C. Briefly, 0.5 mL of solution was introduced to the plate and the cone was rotated at eight different speeds ranging from 0.5 to 100 rpm. After equilibration at each speed, the shear stress, viscosity, and % torque (percent deflection of the cone from neutral position) were recorded. Sample pH (model 320, Corning Inc., Corning, NY) and osmolarity (model 5004, Precision Systems, Inc., Natick, MA) were also recorded at this time.

ANIMAL STUDIES: The rat cecal abrasion model developed by Burns *et al.* has been demonstrated to provide reproducible measurement of efficacy in adhesion prevention.

This is an approved animal procedure and all ethical guidelines were followed. Surgeries and post-operative evaluations were performed by Dr. Lynn Peck. Briefly, female Sprague-Dawley rats weighing 215-265 grams were anesthetized by injection. A laparotomy was performed and approximately 5 mL of PBS (control) or test solution was introduced into the abdominal cavity. The cecum was exposed and four sites abraded by a 1 cm diameter gauze covered probe. This probe is a sliding shaft that applies a 70 gram load while rotated for 60 revolutions per site at 140 rpm. The gauze on the probe was coated with either PBS or test solution and changed between animals.

After abrasion, the laparotomy was closed with sutures and staples. The rats were sacrificed on day 7 post surgery and evaluated for severity (grade 1 to 4) and location of adhesions. Test solutions were compared to PBS on the basis of the incidence of severe adhesions (# of animals with adhesions of grade 2 or higher/# animal in the group); the incidence of no adhesion (# of animals with no adhesions/# animals in the group); and the mean incidence of adhesions (# of adhesions of all grades/# animals in the group). Statistical analysis (GraphPad Instat Mac, GraphPad Software, San Diego, CA) compared the PBS treated (control)

group to the test solution treated using Chi square or Kruskall-Wallis, as appropriate, with a cutoff of p = 0.001.

A.1.3 Results and Discussion

SOLUTION ANALYSIS: The results for the solution analysis on the several solutions used in the animal studies are shown in Table A.1. Viscosity for the PBS control solutions was below the detection limit for the viscometer and cones used, but is presumed to be 1 cps.

Table A.1 Results of solution analyses done on the solutions used in the animal studies. "Low shear" and "high shear" viscosity were performed with a #40 cone at 3.75/sec. and a #52 cone at 100.0/sec. shear rate, respectively.

Solution Type	**Viscosity (low shear)**	**Viscosity (high shear)**	**pH**	**Osmolarity**
PBS (1st study)	---	---	7.08	287 mOsm
0.4% HA	386 cps	128 cps	7.24	281 mOsm
0.9% PEG	426 cps	112 cps	6.91	302 mOsm
1.1% f-PEG	346 cps	414 cps	7.03	299 mOsm
PBS (2nd study)	---	---	7.11	279 mOsm
0.7% CMC	406 cps	161 cps	7.04	306 mOsm
1.1% f-PEG	484 cps	442 cps	7.07	294 mOsm

As can be seen from the table, the unmodified linear polymers (HA, CMC, and PEG) undergo shear thinning as shear rate increases. The f-PEG however does not undergo shear thinning in the shear range examined here. As shown in Figure A.2, the f-PEG solutions appear to undergo shear thickening up to approximately 150/sec. shear rate and then start shear thinning.

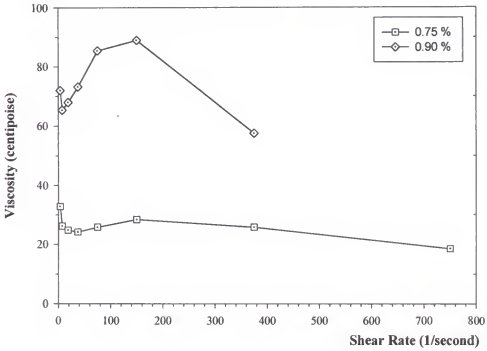

Figure A.2 Viscosity versus shear rate for C8F17 terminated PEG at 0.75% and 0.9% w/v in PBS (#40 cone, 25°C).

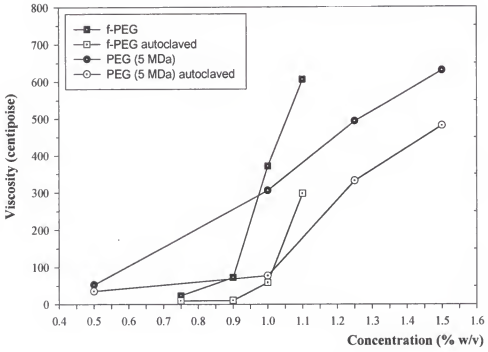

Figure A.3 Viscosity versus concentration for both autoclaved and non-autoclaved PEG (5 MDa) and C8F17 terminated PEG (35 kDa); nominal shear rate 38/sec. at 25°C.

Figure A.3 shows the viscosity versus concentration for the f-PEG solutions versus the high molecular weight PEG solutions. There are several interesting things to note from this figure. First, even though the perfluoroalkane terminated PEG is nearly 1/140th of the molecular weight of the PEG used, these solutions have similar viscosities at concentrations between 0.9% and 1.1% w/v. Next, the effect of concentration on viscosity is much more pronounced for perfluorooctane terminated PEG than for unmodified PEG. When unmodified, 5 MDa PEG has a viscosity of 630 cps at a

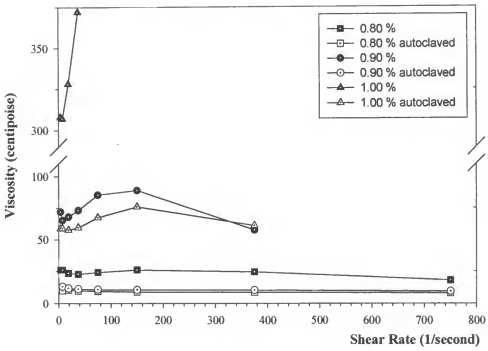

Figure A.4 Autoclaving induced degradation in viscous behavior of C8F17 terminated PEG (35 kDa) for 0.8%, 0.9% and 1.0% w/v in PBS (#40 cone, 25°C).

concentration of 1.5% w/v, the f-PEG has a viscosity of 2260 cps at 1.5% w/v. Finally, the effect of autoclaving can be seen. Treatment of PEG solutions at 121°C and one atmosphere pressure causes degradation in the viscous behavior.

This degradation is considerably more pronounced for the f-PEG as would be expected. Scission of the bifunctional chain does not impair phase separation of the perfluoroalkane end groups, but does disrupt the "gelation" of the solution. The extent of this viscosity reduction is

shown in Figure A.4, which compares the viscosity versus shear rate for three different concentrations of perfluorooctane terminated PEG. Even at the lowest concentration, a 50% reduction in viscosity was seen.

ANIMAL STUDIES: Two separate animal studies were performed which contained 1.1% f-PEG solution among the test solutions. Surgeries were performed over several days for each study, due to the size of the studies; samples were randomized over the course of each study. PBS was used as the negative control in both studies. For positive controls, 0.4% HA (supplied courtesy of Genzyme Corp., Cambridge, MA) was used in the first study and 0.7% CMC was used in the second.

In the first study the 0.9% w/v high molecular weight PEG was also tested. The first five animals tested died within 6 hours of surgery. From the post study report by Peck *et al.*, "[The] Animals developed hematuria within an hour or two of receiving the [PEG] solution. Respiratory distress followed . . . necropsy showed kidneys which appeared swollen or edematous. The livers were dark red. Ecchymotic hemorrhage was seen in the lungs . . ." (55, p. 1). Overall, this is consistent with the findings of Bruns *et al.*[12]

The results for both of the studies are shown in Table A.2.[55, 56] Both the positive controls and the 1.1% f-PEG solutions perform significantly ($p \leq 0.001$) better than PBS. The 1.1% f-PEG solutions give similar if slightly worse performance than either 0.4% HA or 0.7% CMC.

Table A.2 Results of the cecal abrasion studies comparing 1.1% f-PEG solutions to PBS (negative control), 0.4% HA and 0.7% CMC (positive controls) for various measures of adhesion incidence as defined in the text.

Study I Treatments	**% ≥ Grade 2**	**% 0 Incidence**	**Mean Incidence X ± SEM**
PBS	80% (8/10)[a]	10% (1/10)[a]	2.1 ± 0.5 (21/10)[b]
0.4% HA	10% (1/10)	80% (8/10)	0.4 ± 0.3 (4/10)
1.1% f-PEG	10% (1/10)	70% (7/10)	0.4 ± 0.2 (4/10)
Study II Treatments			
PBS	70% (7/10)[a]	30% (3/10)[a]	2.1 ± 0.7 (21/10)[b]
0.7% CMC	13% (1/8)	75% (6/8)	0.4 ± 0.3 (3/8)
1.1% f-PEG	33% (3/9)	67% (6/9)	0.3 ± 0.2 (3/9)

a) Control differs significantly from test solutions, $p \leq 0.001$, by Chi Square.
b) Control differs significantly from test solutions, $p \leq 0.001$, by Kruskal-Wallis.

A.1.4 Conclusions

It has been demonstrated that low molecular weight f-PEG can be used to make low concentration solutions of the viscosity desirable for adhesion prevention solutions. These f-PEG's can be obtained in very pure form, so residual monomer toxicity is significantly reduced. As well, the perfluoroalkane end groups may provide some protection against "unzipping" of the PEG molecule, reducing toxicity from PEG degradation products. These solutions have an interesting shear thinning characteristic which can be attributed to the finite time required to re-establish phase separation "cross links" after shear cleavage.

The use of phase separation to create shear labile gelation of water-soluble polymers should be applicable to other perfluoroalkane/polymer systems, e.g. f-CMC or f-HA; as well as to other hydrophobic end groups, e.g. perchloroalkanes. Even staying within the f-PEG system, it should be possible to tailor the shear thinning characteristics of these solutions. This could be done by changing the size of the perfluoroalkane end group; mixing f-PEG's terminated with different size end groups; or by adding perfluoroalkane terminated PEG star polymers in with the linear f-PEG solution.

A.2 Silicone Breast Implant Gels

A.2.1 Introduction

Since the FDA removed silicone gel filled breast implants from the market in the United States, there has been increasing interest in evaluating these devices. While wide spread concern about the possible adverse immunological impact of these implants has taken the spot light, there are a number of other possible problems with these implants, e.g. capsular formation/contraction and implant rupture, that are worthy of investigation.

Silicone, or polydimethylsiloxane (PDMS), is comprised of a alternating silicon ether linkage backbone with methyl side groups. This material is relatively chemically inert and remains flexible even at extremely low temperatures. The silicone used in these implants comes in three general types. First, the outer shell of almost every breast implant made is composed of densely cross linked PDMS extended with fumed silica (added to impart tear strength). The filling of implants now banned in the United States is also made from silicone. This "gel" is a mixture of lightly cross linked PDMS swollen with very low molecular weight PDMS oil (the other two types of silicone). Very little has

been done to characterize this gel and to that end, a reproducible means of evaluating the flow properties of the silicone gel is needed.

Gel flow properties of silicone breast implants have been measured by ASTM method F703-81, "Standard Specification for Implantable Breast Prostheses", subsection "Test for Gel Cohesion".[8] This is a crude technique in which a relatively large amount of gel (>100 mL) is placed in a metal funnel of defined geometry and allowed to flow out the bottom. This is a pass/fail test wherein to pass it is specified that the gel should not separate nor extend more than 4.5 cm from the bottom of the funnel after 30 minutes at room temperature.

The qualitative, pass/fail nature of this method does not permit any quantitative analysis or comparison of different samples. Conventional methods for measuring the rheological properties of such materials (e.g. rotating spindle or cone/plate rheometers) are unreliable for use with gels such as these silicone gels because the elastic component of gel flow dominates the viscous component.

A melt flow indexer is a widely used instrument for measuring the flow properties of polymer at various temperatures relevant to molding processes.[66] This study

was undertaken to develop a method based on the melt flow indexer to measure the flow properties of silicone gel at physiologic temperature. This method provided accurate and reproducible measurements using as little as 6 mL of material.

A.2.2 Materials and Methods

SAMPLE PREPARATION: Silicone gel was obtained from four unused implants and 22 explanted implants (explants). The explants were obtained in matched pairs and were sterilized by exposure to ethylene oxide gas prior to gel recovery. After thorough visual examination, specimens of the elastomer shell were carefully dissected to obtain samples for other studies. The implant gel was removed and stored in separate, clean, polypropylene containers at room temperature.

GEL FLOW INDEX: The flow index of gel samples (GFI) from each implant was obtained using a Tinius-Olsen, model MP 993 extrusion plastometer (Tinius-Olsen, Inc., Willow Grove, PA). Briefly, samples were introduced into a heated metal tube with a small exit orifice. A standard load was then applied. The temperature was maintained at 37°C and the total ram load was 0.325 kg. The extrudate was

collected at intervals of either 30 or 60 seconds and weighed to the nearest mg. The GFI was calculated as the mass of extrudate divided by flow time and is reported in units of "grams/10 minutes".

A modified orifice with the same exit diameter and path length as the standard orifice supplied with the instrument was used. The modified orifice extends beyond the thermal jacket of the instrument, making it easier to collect the very adhesive gel at each time interval. Comparative runs were done using the same gel with both orifices and no statistical difference could be found in the measured values.

For each gel sample, ten aliquots of extrudate were collected. The data presented are the mean GFI plus/minus the sample standard deviation. Higher values of GFI indicate more fluid (less viscous) gels.

A.2.3 Results and Discussion

The results of gel flow analysis and implant duration are shown in Table A.3 for four control (unimplanted) implants and 22 explants. Implant duration is defined as the time (to the nearest whole year) between implantation and explantation. As seen here, the mean gel flow index

ranges from 0.3 to 13.8 g/10min., with the majority of specimens exhibiting a mean gel flow index between 4.0 and 12.0 g/10min. The implant durations cover 0 to 19 years.

Table A.3 Results for the gel flow analysis of silicone gel filled breast implants including two series of both unused and explanted implants. The gel flow index is given as the mean of ten measurements +/- the sample standard deviation.

SERIES I: Dow Corning Implants			**SERIES II: McGhan Implants**		
Sample	**Implant Duration (Years)**	**Gel Flow Index (g/10min)**	**Sample**	**Implant Duration (Years)**	**Gel Flow Index (g/10min)**
Cont. 1	0	3.4 ± 0.6	Cont. 15	0	7.6 ± 1.6
Cont. 2	0	11.1 ± 1.3	11A	16	4.6 ± 0.9
Cont. 3	0	10.3 ± 1.7	11B	16	6.1 ± 1.3
1A	10	13.0 ± 2.1	12A	13	11.7 ± 2.9
1B	10	11.7 ± 3.0	12B	13	12.5 ± 3.7
2A	19	0.3 ± 0.2	13A	11	2.2 ± 0.4
2B	19	0.4 ± 0.3	13B	11	2.7 ± 0.9
3A	6	10.2 ± 1.6	14A	15	8.6 ± 1.5
3B	6	13.8 ± 2.7	14B	15	7.0 ± 1.6
4A	5	11.4 ± 2.0	15A	12	6.0 ± 0.9
4B	5	10.1 ± 2.3	15B	12	6.4 ± 1.5
5A	15	8.9 ± 1.0	16A	11	4.6 ± 1.2
5B	15	2.4 ± 2.2	16B	11	2.3 ± 0.8

Figure A.5 shows the gel flow index for Dow Corning implants (series I) versus implant duration. Likewise, Figure A.6 shows the gel flow index for McGhan implants (series II) versus implant duration. Predictably, there was low correlation ($R^2 < 0.4$) between the gel flow measurements and the implant duration.

This is to be expected because these implants came from manufacturing lots that span nearly two decades. It is known that specifications for the consistency of the gel varied over the history of individual product lines.

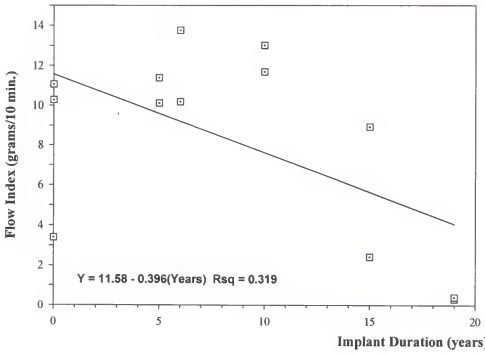

Figure A.5 Gel flow index versus implant duration (to the nearest whole year) for Dow Corning implants, series I.

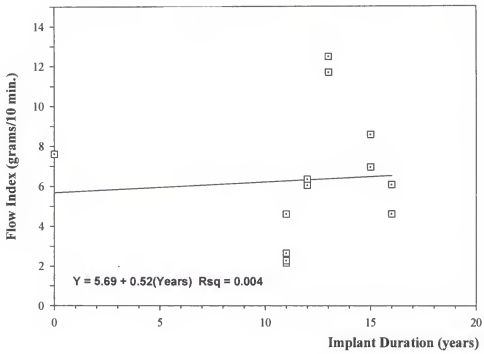

Figure A.6 Gel flow index versus implant duration (to the nearest whole year) for McGhan implants, series II.

A.2.4 Conclusions

So called silicone "gel" is actually a complex, highly elastic, mixture of a minor amount of lightly cross linked PDMS swollen with low molecular weight PDMS oils. The method developed here provides a precise and reliable measurement of breast implant silicone gel flow properties at physiologic temperature. Such measured values will enable quantitative analysis and statistical comparison of

explanted devices. Given the wide variety of implants made relative to the total number implanted, many more samples will have to be analyzed by this method before a widely usable database can be constructed.

In addition to the implant duration, the overall age of the implant and the specifications of the implant manufacturing lot must be examined. Studies are underway to determine the PDMS oil content of the gels via soxhlet extraction with chloroform. The results of the extraction study will be correlated with the gel flow index measurements for corresponding gels. Furthermore, as other means of evaluating gel characteristics are developed (e.g. protein and cholesterol contents), these data can be correlated with the gel flow property.

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BIOGRAPHICAL SKETCH

James F. Kirk was born in 1958 in Andalusia, Alabama. He spent his early childhood in Bucks County, Pennsylvania, north of Philadelphia. He completed his elementary through high school education in St. Petersburg, Florida.

Mr. Kirk attended college in Boston. While there he completed both a BS in mechanical engineering (1983) and an MS in materials science and engineering (1986) at the Massachusetts Institute of Technology as well as earning his MBA (1989) at Boston University. While at MIT, he worked on biodegradable polymeric devices for repair of dermal and neural injuries, under the direction of Dr. I.V. Yannas. Mr. Kirk will be awarded his doctorate in materials science and engineering at the University of Florida in August, 1997. While at UF, he worked on biodegradable microspheres for targeted drug delivery, under the direction of Dr. E.P. Goldberg.

Mr. Kirk has both worked in and served as a consultant to industry since 1984, working primarily in the area of production process design and scale-up for biomedical and pharmaceutical products. While in industry, he worked for Biofunction, Inc., Genzyme Corp. and Innovation Support, Inc. While in industry, Mr. Kirk both assisted and was

directly involved with several entrepreneurial ventures and continues to be greatly interested in entrepreneurship.

He has held appointments at MIT as both a lecturer and a visiting lecturer in the Department of Mechanical Engineering where he co-taught a course on entrepreneurship. He also served as a visiting scientist in the Department of Materials Science and Engineering (MIT); where he did research on collagen based prostheses for promoting tissue regeneration.

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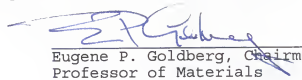

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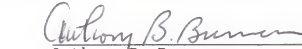

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